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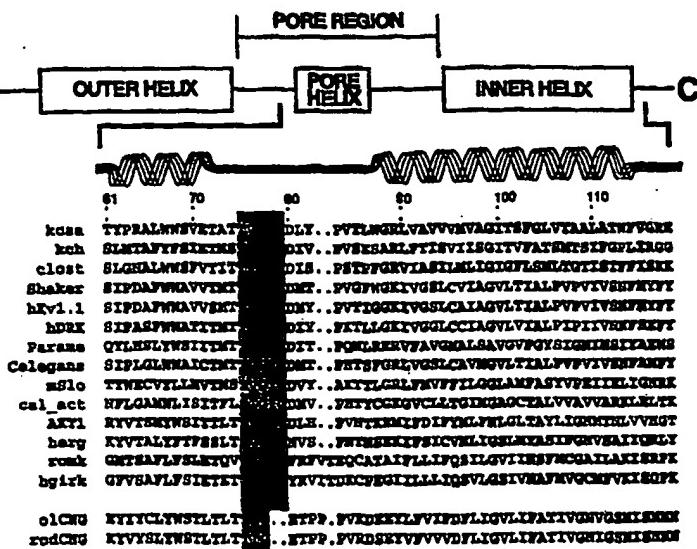
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(54) Title: ASSAYS FOR SCREENING COMPOUNDS WHICH INTERACT WITH CATION CHANNEL PROTEINS, MUTANT PROKARYOTIC CATION CHANNEL PROTEINS, AND USES THEREOF



(57) Abstract

Assays for screening potential drugs or agents that can interact and potentially bind to cation channel proteins, and potentially have uses in treating conditions related to the function of cation channel proteins is provided, along with prokaryotic cation channel proteins mutated to mimic eukaryotic cation channels, which can then be used in assays of the present invention.

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ASSAYS FOR SCREENING COMPOUNDS WHICH INTERACT WITH CATION CHANNEL PROTEINS, MUTANT PROKARYOTIC CATION CHANNEL PROTEINS, AND USES THEREOF

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CROSS REFERENCE TO RELATED APPLICATIONS

This Application is a continuation in part of an Application filed on March 20, 1998, entitled "Assays for Screening Compounds which Interact with Cation Channel Proteins, Mutant Prokaryotic Cation Channel Proteins, and Uses Thereof."

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GOVERNMENT RIGHTS CLAUSE

The research leading to the present invention was supported in part with National Institutes of Health Grant GM 43949. The government may have rights in the invention.

FIELD OF INVENTION

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The present invention relates to a crystal of a cation channel protein, and methods of using such a crystal in screening potential drugs and therapeutic agents for use in treating conditions related to the function of such channels *in vivo*.

BACKGROUND OF INVENTION

20

Although numerous types of channel proteins are known, the main types of ion channel proteins are characterized by the method employed to open or close the channel protein to either permit or prevent specific ions from permeating the channel protein and crossing a lipid bilayer cellular membrane. One important type of channel protein is the voltage-gated channel protein, which is opened or closed (gated) in response to changes in electrical

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potential across the cell membrane. Another type of ion channel protein are called mechanically gated channel proteins, for which a mechanical stress on the protein opens or closes the channel. Still another type is called a ligand-gated channel, which opens or closes depending on whether a particular ligand is bound the protein. The ligand can be either an extracellular moiety, such as a neurotransmitter, or an intracellular moiety, such

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as an ion or nucleotide.

Presently, over 100 types of ion channel proteins have been described, with additional ones being discovered. Basically, all ion channels have the same basic structure regarding the permeation of their specific ion, although different gating mechanisms (as described above) can be used. One of the most common types of channel proteins, found in the membrane of almost all animal cells, permits the specific permeation of potassium ions (K^+) across a cell

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membrane. In particular, potassium ions permeate rapidly across cell membranes through K⁺ channel proteins (up to 10⁸ ions per second). Moreover, potassium channel proteins have the ability to distinguish among potassium ions, and other small alkali metal ions, such as Li⁺ or Na⁺ with great fidelity. In particular, potassium ions are at least ten thousand times more permeant than sodium ions. In light of the fact that both potassium and sodium ions are generally spherical in shape, with radii of about 1.33 Å and 0.95 Å respectively, such selectivity is remarkable.

Broadly, potassium channel proteins comprise four (usually identical) subunits. Presently 10 two major types of subunits are known. One type of subunit contains six long hydrophobic segments (presumably membrane-spanning), while the other type contains two hydrophobic segments. Regardless of what type of subunits are used, potassium channel proteins are highly selective for potassium ions, as explained above.

15 Among their many functions, potassium channel proteins control the pace of the heart, regulate the secretion of hormones such as insulin into the blood stream, generate electrical impulses underlying information transfer in the nervous system, and control airway and vascular smooth muscle tone. Thus, potassium channels participate in cellular control processes that are abnormal, such as cardiac arrhythmia, diabetes mellitus, seizure disorder, asthma and hypertension, to name only a few.

20 Although potassium channel proteins are involved in such a wide variety of homeostatic functions, few drugs or therapeutic agents are available that act on potassium channel proteins to treat abnormal processes. A reason for a lack of presently available drugs that 25 act on potassium channel proteins is that isolated potassium channel proteins are not available in great abundance, mainly because an animal cell requires only a very limited number of such channel proteins in order to function. Consequently, it has been very difficult to isolate and purify potassium channel proteins, reducing the amount of drug screening efforts in search of potassium channel protein acting drugs.

30 Hence, what is needed is accurate information regarding the structure of cation channel proteins so that drugs or therapeutic agents having an appropriate structure to potentially interact with a cation channel protein can be selected.

What is also needed is an ability to overcome the physical limitations regarding the isolation and purification of cation channel proteins, particularly potassium ion channel proteins.

- 5 What is also needed is a reliable method of utilizing cation channel proteins in screening potential drugs or agents for their possible use in treating conditions related to the function of cation channel proteins *in vivo*.

- What is also needed are novel methods of using accurate information regarding the
10 structure of cation channel proteins so that drugs or therapeutic agents can be screened for potential activity in treating abnormal control processes of the body.

The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

15

SUMMARY OF THE INVENTION

- There is provided, in accordance with the present invention, a method of preparing a functional cation channel protein for use in an assay for screening potential drugs or other agents which interact with a cation channel protein, which permits the screening of
20 potential drugs or agents that may be used as potential therapeutic agents in treating conditions related to the function of cation channel proteins *in vivo*.

- More specifically, the method comprising the steps of providing a functional cation channel protein, conjugating the functional cation channel protein to a solid phase resin, contacting
25 the potential drug or agent to the functional cation channel protein conjugated to the solid phase resin, removing the functional cation channel protein from the solid phase resin, and determining whether the potential drug or agent is bound to the cation channel protein.

- In particular, the present invention extends to a method of preparing a functional cation
30 channel protein for use in an assay as described above, wherein the providing step of the method comprises expressing an isolated nucleic acid molecule encoding the cation channel protein in a unicellular host, such that the cation channel protein is present in the cell membrane of the unicellular host, lysing the unicellular host in a solubilizing solution so that the cation channel protein is solubilized in the solution, and extracting the cation

channel protein from the solubilizing solution with a detergent. In a preferred embodiment, the isolated nucleic acid molecule comprises a DNA sequence of SEQ ID NO:17, or degenerate variants thereof, or an isolated nucleic acid molecule hybridizable under standard hybridization conditions to an isolated nucleic acid molecule having a DNA sequence of SEQ ID NO:17, or degenerate variants thereof.

Numerous methods of lysing a unicellular host are known to the skilled artisan, and have applications in the present invention. In a preferred embodiment, lysing the unicellular host in a solubilizing solution comprises sonicating the unicellular host in a protein solubilizing solution comprising 50 mM Tris buffer, 100 mM KCl, 10 mM MgSO₄, 25 mg DNase 1, 250 mM sucrose, pepstatin, leupeptin, and PMSF, pH 7.5.

Furthermore, a skilled artisan is aware of numerous detergents that can be used to extract an integral membrane bound protein, such as a cation channel protein, from a solubilizing solution described above. Examples of such detergents include SDS, Triton-100, Tween 20, Tween 80, glycerol, or decylmaltoside, to name only a few. Preferably, 40 mM decylmaltoside is used to extract the cation channel protein from the solubilizing solution.

Moreover, numerous solid phase resins to which a functional cation channel protein can be conjugated have applications in a method of preparing a functional cation channel protein for use in an assay, as described above. For example, a solid phase resin comprising insoluble polystyrene beads, PVDF, polyethylene glycol, or a cobalt resin, to name only a few have application in the present invention. Preferably, a cation channel protein is conjugated to a cobalt resin at a protein to resin ratio that allows for saturation of the resin with the cation channel protein. Moreover, after conjugation, the cobalt resin is preferably used to line a column having a volume of about 1 ml.

After the cation channel protein is conjugated to a solid phase resin, it is contacted with a potential drug or agent, which is given an opportunity to bind to the cation channel protein.

Subsequently, the cation channel protein is removed from the solid phase resin, and analyzed to determine whether the potential drug or agent is bound thereto. Numerous methods of removing the cation channel protein from the solid phase resin are known to those of ordinary skill in the art. In a preferred embodiment, wherein the solid phase resin

is a cobalt resin, the removing step comprises contacting the cation channel protein conjugated to the solid phase resin with an imidazole solution. This solution readily cleaves any bonds conjugating the cation channel protein to the resin, so that the protein can be removed from the resin, and collected for further analysis to determine whether the potential drug or agent is bound to the protein.

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After the cation channel protein has been removed from the resin, it must be examined to determine whether the potential drug or agent is bound thereto. If bound, the drug or agent may have uses involved in modulation of the function of a cation channel protein *in vivo*,
10 including uses as a therapeutic agent in treating conditions related to the function of cation channel proteins. Numerous analytical methods are presently available to the skilled artisan for determining whether the potential ligand is bound to the cation channel protein. Examples of such methods include molecular weight analysis with SDS-PAGE, immunoassays using an antibody to the drug or agent, HPLC, or mass spectrometry.

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Furthermore, the present invention extends to a method of using a functional cation channel protein in an assay for screening potential drugs or agents which interact with the cation channel protein, wherein the potential drug or agent is a member of a library of compounds, which is contacted to the cation channel protein. Examples of libraries having
20 applications in the present invention include, but are not limited to, a mixture of compounds, or a combinatorial library of compounds. Furthermore, examples of combinatorial compounds having applications in the present invention include, but are not limited to, a phage display library, or a synthetic peptide library, to name only a few.

25 In another embodiment, the present invention extends to a prokaryotic cation channel protein mutated to mimic a functional eukaryotic cation channel protein. More specifically, Applicant has discovered that all cation channel proteins from all organisms have a conserved structure. Hence, placing mutations in a potassium channel from a prokaryotic organism, for example, can permit the use of the prokaryotic cation channel protein in
30 screening assays for drugs that may interact with specific eukaryotic cation channel proteins. For example, a prokaryotic potassium channel protein can be mutated to mimic a cardiac potassium channel protein, a venous potassium channel protein, or a neuro potassium channel of a human, to name only a few.

Hence, pursuant to the present invention, a prokaryotic potassium channel protein, a prokaryotic sodium channel protein, or a prokaryotic calcium channel protein can be mutated to mimic a eukaryotic cation channel protein.

- 5 Examples of prokaryotic organisms from which a prokaryotic cation channel protein can be taken and mutated to mimic a eukaryotic cation channel protein include *E.coli*, *Streptomyces lividans*, *Clostridium acetobutylicum*, or *Staphylococcus aureus*, to name only a few. Furthermore, such prokaryotic cation channel proteins can comprise an amino acid sequence of SEQ ID Nos: 1, 2, 3, or 7, or conserved variants thereof. In a preferred 10 embodiment, the prokaryotic cation channel protein mutated to mimic a eukaryotic cation channel protein, wherein the prokaryotic cation channel protein is a potassium channel protein from *Streptomyces lividans*.

Furthermore, pursuant to the present invention, a prokaryotic cation channel protein can be 15 mutated to mimic eukaryotic potassium channel protein, a eukaryotic sodium channel protein, or a eukaryotic calcium channel protein. Preferably, the eukaryotic cation channel protein is produced endogenously in a eukaryotic organism, such as an insect or a mammal, for example. More specifically, pursuant to the present invention, a prokaryotic cation channel protein is mutated to mimic a eukaryotic cation channel protein endogenously 20 produced in a eukaryotic organism selected from the group consisting of *Drosophila melanogaster*, *Homo sapiens*, *C. elegans*, *Mus musculus*, *Arabidopsis thaliana*, *paramecium tetraurelia* or *Rattus norvegicus*, or having an amino acid sequence comprising SEQ ID Nos: 4, 5, 6, 8, 9, 10, 11, 12, 13, or 14, or conserved variants thereof.

- 25 In a preferred embodiment, the present invention extends to a prokaryotic cation channel protein mutated to mimic a functional eukaryotic channel protein, wherein the prokaryotic cation channel protein is a potassium channel protein from *Streptomyces lividans* comprising an amino acid sequence of SEQ ID NO:1 or degenerate variants thereof, and 30 the eukaryotic cation channel is a potassium channel protein comprising an amino acid sequence of SEQ ID NO:4 or conserved variants thereof. As a result, the mutated prokaryotic channel protein comprises an amino acid sequence of SEQ ID NO:16, or conserved variants thereof, which is encoded by an isolated nucleic acid molecule comprising a DNA sequence of SEQ ID NO:17, or degenerate variants thereof.

- In another embodiment, the present invention extends to a method of using a crystal of a cation channel protein, as described herein, in an assay system for screening drugs and other agents for their ability to modulate the function of a cation channel protein, comprising the steps of initially selecting a potential drug or agent by performing rational drug design with the three-dimensional structure determined for a crystal of the present invention, wherein the selecting is performed in conjunction with computer modeling.
- 5 After potential drugs or agents have been selected, a cation channel protein is contacted with the potential drug or agent. If the drug or therapeutic agent has potential use for modulating the function of a cation channel protein, a change in the function of the cation channel after contact with the agent, relative to the function of a similar cation channel protein not contacted with the agent, or the function of the same cation channel protein prior to contact with the agent. Hence, the change in function is indicative of the ability of the drug or agent to modulate the function of a cation channel protein.
- 10
- 15 Furthermore, the present invention extends to a method of using a crystal of a cation channel protein as described herein, in an assay system for screening drugs and other agents for their ability to modulate the function of a cation channel protein, wherein the crystal comprises a Na^+ channel protein, a K^+ channel protein, or a Ca^{2+} channel protein.
- 20 The present invention further extends to a method of using a crystal of a cation channel protein in an assay for screening drugs or other agents for their ability to modulate the function of a cation channel protein, wherein the crystal of the cation channel protein comprises an amino acid sequence of:
- 25 residues 23 to 119 of SEQ ID NO:1 (*Streptomyces lividans*);
residues 61 to 119 of SEQ ID NO:2 (*E. coli*);
residues 61 to 119 of SEQ ID NO:3 (*Clostridium acetobutylicum*);
residues 61 to 119 of SEQ ID NO:4 (*Drosophila melanogaster*);
residues 61 to 119 of SEQ ID NO:5 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:6 (*Homo sapiens*);
30 residues 61 to 119 of SEQ ID NO:7 (*Paramecium tetraurelia*);
residues 61 to 119 of SEQ ID NO:8 (*C. elegans*);
residues 61 to 119 of SEQ ID NO:9 (*Mus musculus*);
residues 61 to 119 of SEQ ID NO:10 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:11 (*Arabidopsis thaliana*);

residues 61 to 119 of SEQ ID NO:12 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:13 (*Rattus norvegicus*); or
residues 61 to 119 of SEQ ID NO:14 (*Homo sapiens*);
or conserved variants thereof.

5

In a preferred embodiment of a method of using a crystal of a cation channel protein in an assay for screening drugs or other agents for their ability to modulate the function of a cation channel protein, the crystal comprises a potassium channel protein, comprising amino acid residues 23 to 119 of SEQ ID NO:1, a space grouping of C2, and a unit cell of 10 dimensions of $a=128.8 \text{ \AA}$, $b=68.9\text{\AA}$, $c=112.0 \text{ \AA}$, and $\beta=124.6^\circ$.

Moreover, it is important to note that a drug's or agent's ability to modulate the function of a cation channel protein includes, but is not limited to, increasing or decreasing the cation channel protein's permeability to the specific cation relative the permeability of the same 15 or a similar not contacted with the drug or agent, or the same cation channel protein prior to contact with the drug or agent.

In a further embodiment, the present invention extends to a method of using a crystal of a cation channel protein, as set forth herein, in an assay system for screening drugs and other 20 agents for their ability to treat conditions related to the function of cation channel proteins *in vivo*, and particularly in abnormal cellular control processes related to the functioning of cation channel protein. Such a method comprises the initial step of selecting a potential drug or other agent by performing rational drug design with the three-dimensional structure determined for a crystal of the invention, wherein the selecting is performed in conjunction 25 with computer modeling. After potential drugs or therapeutic agents are selected, a cation channel protein is contacted with the potential drug or agent. If an interaction of the potential drug or other agent with the cation channel is detected, it is indicative of the potential use of the drug or agent to treat conditions related the function of cation channel proteins *in vivo*. Examples of such conditions include, but are not limited to, cardiac 30 arrhythmia, diabetes mellitus, seizure disorder, asthma or hypertension, to name only a few.

Furthermore, a crystal of a cation channel protein used in the method for screening drugs or agents for their ability to interact with a cation channel comprises an Na^+ channel

protein, K⁺ channel protein, or Ca²⁺ channel protein. Hence, the method of the present invention can be used to screen drugs or agents capable of treating conditions related to the function of such channels.

- 5 Moreover, the present invention extends to a crystal used in the method for screening drugs or agents for their ability to interact with a cation channel protein comprising an amino acid sequence of:

residues 23 to 119 of SEQ ID NO:1 (*Streptomyces lividans*);
residues 61 to 119 of SEQ ID NO:2 (*E. coli*);
10 residues 61 to 119 of SEQ ID NO:3 (*Clostridium acetobutylicum*);
residues 61 to 119 of SEQ ID NO:4 (*Drosophila melanogaster*);
residues 61 to 119 of SEQ ID NO:5 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:6 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:7 (*Paramecium tetraurelia*);
15 residues 61 to 119 of SEQ ID NO:8 (*C. elegans*);
residues 61 to 119 of SEQ ID NO:9 (*Mus musculus*);
residues 61 to 119 of SEQ ID NO:10 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:11 (*Arabidopsis thaliana*);
residues 61 to 119 of SEQ ID NO:12 (*Homo sapiens*);
20 residues 61 to 119 of SEQ ID NO:13 (*Rattus norvegicus*); or
residues 61 to 119 of SEQ ID NO:14 (*Homo sapiens*),

or conserved variants thereof.

In a preferred embodiment, a crystal used in a method for screening drugs or agents for 25 their ability to interact with a cation channel, comprises amino acid residues 23 to 119 of SEQ ID NO:1, has a space grouping of C2, and a unit cell of dimensions of a=128.8 Å, b=68.9 Å, c=112.0 Å, and β=124.6°.

In yet another embodiment, the present invention extends to a method of using a crystal of 30 a cation channel protein described herein, in an assay system for screening drugs and other agents for their ability to permeate through a cation channel protein, comprising an initial step of selecting a potential drug or other agent by performing rational drug design with the three-dimensional structure determined for the crystal, wherein the selecting of the potential drug or agent is performed in conjunction with computer modeling. After a potential drug

or agent has been selected, a cation channel protein can be prepared for use in the assay. For example, preparing the cation channel protein can include isolating the cation channel protein from the membrane of a cell, and then inserting the cation channel protein into a membrane having a first and second side which is impermeable to the potential drug or agent. As a result, the cation channel protein traverses the membrane, such that the extracellular portion of the cation channel protein is located on the first side of the membrane, and the intracellular portion of the cation channel protein is located on the second side of the membrane. The extracellular portion of the cation channel membrane can then be contacted with the potential drug or agent. The presence of the drug or agent in the second side of the membrane is indicative of the drug's or agent's potential to permeate the cation channel protein, and the drug or agent is selected based on its ability to permeate the cation channel protein.

In addition, a crystal used in a method for screening drugs or agents for their ability to permeate a cation channel can comprise a Na⁺ channel protein, a K⁺ protein channel, or a Ca²⁺ protein channel.

Furthermore, the present invention extends to the use of a crystal in an assay system for screening drugs and other agents for their ability to permeate through a cation channel protein, wherein the crystal comprises an amino acid sequence of:

residues 23 to 119 of SEQ ID NO:1 (*Streptomyces lividans*);
residues 61 to 119 of SEQ ID NO:2 (*E. coli*);
residues 61 to 119 of SEQ ID NO:3 (*Clostridium acetobutylicum*);
residues 61 to 119 of SEQ ID NO:4 (*Drosophila melanogaster*);
residues 61 to 119 of SEQ ID NO:5 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:6 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:7 (*Paramecium tetraurelia*);
residues 61 to 119 of SEQ ID NO:8 (*C. elegans*);
residues 61 to 119 of SEQ ID NO:9 (*Mus musculus*);
residues 61 to 119 of SEQ ID NO:10 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:11 (*Arabidopsis thaliana*);
residues 61 to 119 of SEQ ID NO:12 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:13 (*Rattus norvegicus*); or
residues 61 to 119 of SEQ ID NO:14 (*Homo sapiens*);

or conserved variants thereof.

In a preferred embodiment, the crystal used in an assay system of the present invention for screening drugs and other agents for their ability to permeate through a cation channel
5 protein comprises amino acid residues 23 to 119 of SEQ ID NO:1, has a space grouping of C2, and a unit cell of dimensions of $a=128.8 \text{ \AA}$, $b=68.9\text{\AA}$, $c=112.0 \text{ \AA}$, and $\beta=124.6^\circ$.

Naturally, the present invention extends to an isolated nucleic acid molecule encoding a mutant K⁺ channel protein, comprising a DNA sequence of SEQ ID NO:17, or degenerate
10 variants thereof.

Furthermore, the present invention extends to an isolated nucleic acid molecule hybridizable to an isolated nucleic acid molecule encoding a mutant K⁺ channel protein under standard hybridization conditions.

15 Moreover, isolated nucleic acid molecules of the present invention, and described above, can be detectably labeled. Examples of detectable labels having applications in the present invention include, but are not limited to, radioactive isotopes, compounds which fluoresce, or enzymes.

20 The present invention further extends to an isolated nucleic acid molecule encoding a mutant K⁺ channel protein, or degenerate variants thereof, comprising an amino acid sequence of SEQ ID NO:16, or conserved variants thereof.

25 In addition, the present invention extends to an isolated nucleic acid molecule encoding a polypeptide comprising an amino acid sequence of SEQ ID NO:16, or conserved variants thereof, wherein the isolated nucleic acid molecule is hybridizable under standard hybridization conditions to an isolated nucleic acid molecule encoding a K⁺ channel protein, or degenerate variants thereof.

30 Furthermore, the present invention extends to a mutant cation channel protein comprising an amino acid sequence of SEQ ID NO:16, or conserved variants thereof.

In addition, the present invention extends to a cloning vector comprising an isolated nucleic

acid molecule, or degenerate variants thereof, which encodes a mutant cation channel protein of the present invention, or conserved variants thereof, and an origin of replication. The present invention also extends to a cloning vector comprising an origin of replication and an isolated nucleic acid molecule hybridizable under standard hybridization conditions to an isolated nucleic acid molecule, or degenerate variants thereof, which encodes a mutant cation channel protein of the present invention.

Examples of cloning vectors having applications in the present invention include, but are not limited to, *E. coli*, bacteriophages, plasmids, and pUC plasmid derivatives. More specifically, examples of bacteriophages, plasmids, and pUC plasmid derivatives having applications herein comprise lambda derivatives, pBR322 derivatives, and pGEX vectors, or pmal-c, pFLAG, respectively.

Naturally, the present invention extends to an expression vector comprising an isolated nucleic acid molecule comprising a DNA sequence of SEQ ID NO:17, or degenerate variants thereof, operatively associated with a promoter. In another embodiment, an expression vector comprises an isolated nucleic acid molecule hybridizable under standard hybridization conditions to an isolated nucleic acid comprising a DNA sequence of SEQ ID NO:17, or degenerate variants thereof, operatively associated with a promoter.

Examples of promoters having applications in expression vectors of the present invention comprise immediate early promoters of hCMV, early promoters of SV40, early promoters of adenovirus, early promoters of vaccinia, early promoters of polyoma, late promoters of SV40, late promoters of adenovirus, late promoters of vaccinia, late promoters of polyoma, the *lac* the *trp* system, the *TAC* system, the *TRC* system, the major operator and promoter regions of phage lambda, control regions of fd coat protein, 3-phosphoglycerate kinase promoter, acid phosphatase promoter, or promoters of yeast α mating factor.

Furthermore, the present invention extends to a unicellular host transformed or transfected with an expression vector of the present invention. Such a unicellular host can be selected from the group consisting of *E. coli*, Pseudonomas, Bacillus, Streptomyces, yeast, CHO, R1.1, B-W, L-M, COS1, COS7, BSC1, BSC40, BMT10 and Sf9 cells.

Naturally, the present invention extends to a method of producing a mutant cation channel

- protein, comprising the steps of culturing a unicellular host transformed or transfected with an expression vector of the present invention under conditions that provide for expression of the isolated nucleic acid molecule of the expression vector and recovering the mutant cation channel protein from the unicellular host. Moreover, such a method can also be
- 5 used wherein the expression vector comprises a an isolated nucleic acid molecule hybridizable under standard hybridization conditions to an isolated nucleic acid molecule comprising a DNA sequence of SEQ ID NO:17, or degenerate variants thereof, operatively associated with a promoter.
- 10 The present invention further extends to an antibody having a mutant cation channel protein of the present invention as an immunogen. More specifically, an antibody of the present invention can be a monoclonal antibody, a polyclonal antibody, or a chimeric antibody. Furthermore, an antibody of the present invention can be detectably labeled. Examples of detectable labels having applications in the present invention include, but are not limited to,
- 15 an enzyme, a chemical which fluoresces, or a radioactive isotope.
- Broadly, the present invention extends to a crystal of a cation channel protein having a central pore, which is found natively in a lipid bilayer membrane of an animal cell, such that the central pore communicates with extracellular matrix and cellular cytosol, wherein
- 20 the crystal effectively diffracts x-rays to a resolution of greater than 3.2 angstroms.
- Moreover, the present invention extends to a crystal of a cation channel protein as described above, wherein the cation channel protein comprises a first layer of aromatic amino acid residues positioned to extend into the lipid bilayer membrane proximate to the
- 25 interface an extracellular matrix and lipid bilayer membrane, a second layer of aromatic amino acid residues positioned to extend into the lipid bilayer membrane proximate to the interface of cellular cytosol and said lipid bilayer membrane, a tetramer of four identical transmembrane subunits, and a central pore formed by the four identical transmembrane subunits.
- 30 Moreover, the present invention extends to a crystal of a cation channel protein described above, wherein each transmembrane subunit comprises an inner transmembrane alpha-helix which has a kink therein, an outer transmembrane alpha-helix, and a pore alpha-helix, wherein each subunit is inserted into the tetramer of the cation channel protein so that the

outer transmembrane helix of each subunit contacts the first and second layers of aromatic amino acid residues described above, and abuts the lipid bilayer membrane. Moreover, the inner transmembrane helix of each subunit abuts the central pore of the cation channel protein, contacts the first and second layers of aromatic amino acid residues, is tilted by about 25° with respect to the normal of the lipid bilayer membrane, and is packed against inner transmembrane alpha helices of other transmembrane subunits at the second layer of aromatic amino acid residues forming a bundle of helices at the second layer. The pore alpha-helix of each subunit is located at the first layer of said aromatic amino acid residues, and positioned between inner transmembrane alpha-helices of adjacent subunits, and are directed, in an amino to carboxyl sense, towards the center of the central pore .

Furthermore, the present invention extends to a crystal described above, comprising a cation channel protein having a central pore, which comprises a pore region located at the first layer of aromatic amino acid residues, and connected to the inner and outer transmembrane alpha-helices of said subunits. More particularly, the pore region comprises about 25–45 amino acid residues, a turret connected to the pore alpha-helix and the outer alpha-helix, wherein turret is located at the interface of said extracellular matrix and the lipid bilayer membrane. The pore region further comprises an ion selectivity filter connected to the pore alpha-helix and the inner transmembrane alpha-helix of each subunit.

The ion selectivity filter extends into the central pore of the cation channel protein, and comprises a signature amino acid residue sequence having main chain atoms which create a stack of sequential oxygen atoms along the selectivity filter that extend into the central pore, and amino acid residues having side chains that interact with the pore helix. It is the signature sequence which enables a cation channel protein to discriminate among the cation intended to permeate the protein, and other cations, so that only the cation intended to permeate the channel protein is permitted to permeate.

The central pore further comprises a tunnel into the lipid bilayer membrane which communicates with the cellular cytosol, and a cavity located within the lipid bilayer membrane between the pore region and the tunnel, and connected to the them, such that the central pore crosses the membrane.

Furthermore, the structure of all ion channel proteins share common features, which are set forth in the crystal of a cation channel protein described above. Consequently, the present

invention extends to a crystal of a cation channel protein having a central pore and structure, as described above, wherein the cation is selected from the group consisting of: Na⁺, K⁺, and Ca²⁺. Hence, the present invention extends to crystals of potassium channel proteins, sodium channel proteins, and calcium ion channels, to name only a few. In a 5 preferred embodiment, the crystal of a cation channel protein comprises a crystal of a potassium ion channel protein.

In addition, a crystal of a cation channel protein of a present invention comprises the amino acid sequence of any presently known, or subsequently discovered cation protein 10 channel. Consequently, the present invention extends to a crystal of a cation channel protein having a central pore, which is found natively in a lipid bilayer membrane of an animal cell, such that the central pore communicates with extracellular matrix and cellular cytosol, wherein the crystal comprises an amino acid sequence of:

residues 23 to 119 of SEQ ID NO:1 (*Streptomyces lividans*);
15 residues 61 to 119 of SEQ ID NO:2 (*E. coli*);
residues 61 to 119 of SEQ ID NO:3 (*Clostridium acetobutylicum*);
residues 61 to 119 of SEQ ID NO:4 (*Drosophila melanogaster*);
residues 61 to 119 of SEQ ID NO:5 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:6 (*Homo sapiens*);
20 residues 61 to 119 of SEQ ID NO:7 (*Paramecium tetraurelia*);
residues 61 to 119 of SEQ ID NO:8 (*C. elegans*);
residues 61 to 119 of SEQ ID NO:9 (*Mus musculus*);
residues 61 to 119 of SEQ ID NO:10 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:11 (*Arabidopsis thaliana*);
25 residues 61 to 119 of SEQ ID NO:12 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:13 (*Rattus norvegicus*); or
residues 61 to 119 of SEQ ID NO:14 (*Homo sapiens*);
or conserved variants thereof.

30 In a preferred embodiment, a crystal of the present invention having a central pore, which is found natively in a lipid bilayer membrane of an animal cell, such that the central pore communicates with extracellular matrix and cellular cytosol, comprises an amino sequence of amino acid residues 23 to 119 of SEQ ID NO:1, has a space grouping of C2, a unit cell of dimensions of a=128.8 Å, b=68.9 Å, c=112.0 Å, and β=124.6°.

Furthermore, the present invention extends to a crystal of a cation channel protein having a central pore, which is found natively in a lipid bilayer membrane of an animal cell, such that the central pore communicates with extracellular matrix and cellular cytosol, wherein the channel protein comprises a signature sequence comprising:

5 Thr-Val-Gly-Tyr-Gly-Asp (SEQ ID NO:15).

In another embodiment, the present invention extends to a method for growing a crystal of a cation channel protein having a central pore, which is found natively in a lipid bilayer membrane of an animal cell, such that the central pore communicates with extracellular
10 matrix and cellular cytosol, by sitting-drop vapor diffusion. Such a method of the present invention comprises the steps of providing the cation channel protein, removing a predetermined number of carboxy terminal amino acid residues from the cation channel protein to form a truncated cation channel protein, dissolving the truncated cation channel protein in a protein solubilizing solution, such that the concentration of dissolved truncated
15 channel protein is about 5 to about 10 mg/ml, and mixing equal volumes of protein solubilizing solution with reservoir mixture at 20 ° C. Preferably, the reservoir mixture comprises 200 mM CaCl₂, 100 mM Hepes, 48 % PEG 400, pH 7.5, and the protein solution comprises (150 mM KCl, 50 mM Tris, 2 mM DTT, pH 7.5).
20 Moreover, the present invention extends to a method of growing a crystal of a cation channel protein as described above, wherein a crystal can be grown comprising any kind of cation channel protein. In particular, the present invention can be used to grow crystals of potassium channel proteins, sodium channel proteins, or calcium channel proteins, to name only a few.

25

Furthermore, the present invention extends to a method of growing a crystal of a cation channel protein, as described herein, wherein the crystal comprises an amino acid sequence of:

residues 23 to 119 of SEQ ID NO:1 (*Streptomyces lividans*);
30 residues 61 to 119 of SEQ ID NO:2 (*E. coli*);
residues 61 to 119 of SEQ ID NO:3 (*Clostridium acetobutylicum*);
residues 61 to 119 of SEQ ID NO:4 (*Drosophila melanogaster*);
residues 61 to 119 of SEQ ID NO:5 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:6 (*Homo sapiens*);

residues 61 to 119 of SEQ ID NO:7 (*Paramecium tetraurelia*);
residues 61 to 119 of SEQ ID NO:8 (*C. elegans*);
residues 61 to 119 of SEQ ID NO:9 (*Mus musculus*);
residues 61 to 119 of SEQ ID NO:10 (*Homo sapiens*);
5 residues 61 to 119 of SEQ ID NO:11 (*Arabidopsis thaliana*);
residues 61 to 119 of SEQ ID NO:12 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:13 (*Rattus norvegicus*); or
residues 61 to 119 of SEQ ID NO:14 (*Homo sapiens*);
or conserved variants thereof.

10

Numerous methods can be used to provide a cation channel protein, for use in growing a crystal. For example, traditional purification techniques such as gel filtration, HPLC, or immunoprecipitation can be used to purify cation channel proteins from the membranes of numerous cells. In another method, recombinant DNA technology can be used, wherein a 15 nucleic acid molecule encoding the particular cation channel protein can be inserted into an expression vector, which is then used to transfect a unicellular host. After transfection, the host can be induced to express the nucleic acid molecule, and the particular cation channel protein can be harvested from the membrane of the unicellular host.

20 Moreover, numerous methods are available for removing a predetermined number of carboxy terminal amino acid residues from the cation channel protein to form a truncated cation channel protein. For example, chemical techniques can be used to cleave a peptide bond between two particular amino acid residues in the carboxy terminus of the cation channel protein. In another embodiment, the cation channel protein can be contacted with a 25 proteolytic enzyme, so that the predetermined number of residues from the carboxy terminus are enzymatically removed from the carboxy terminus of the cation channel protein, forming a truncated cation channel protein. In a preferred embodiment, the cation channel protein comprises a potassium channel protein having an amino acid sequence of SEQ ID NO:1, which is contacted with chymotripsin so that residues 1-22 are removed, 30 forming a truncated potassium channel protein comprising an amino acid sequence of residues 23-119 of SEQ ID NO:1.

This invention further provides for a prescreening method for identifying potential modulators of potassium ion channel function comprising the steps of : (i)binding a soluble

potassium ion channel protein to a solid support where the ion channel has the scaffold of a two-transmembrane-domain-type potassium ion channel and has a tetrameric confirmation; (ii) contacting the soluble potassium ion channel protein of step i with a compound in an aqueous solution; and, (iii) determining the binding of the compound to the soluble 5 potassium ion channel protein.

In addition, this invention provides for a method of screening for compounds which selectively bind to a potassium ion channel protein comprising: (i) complexing a functional two-transmembrane-domain-type potassium ion channel protein to a solid support; (ii) 10 contacting the complexed protein/solid support with an aqueous solution said solution containing a compound that is being screened for the ability to selectively bind to the ion channel protein; and, (iii) determining whether the compound selectively binds to the ion channel protein with the proviso that the potassium ion channel protein is in the form of a tetrameric protein; and, when the protein is mutated to correspond to the agitoxin2 docking 15 site of a Shaker K⁺ channel protein by substituting amino acid residues permitting the mutated protein to bind agitoxin2, the protein will bind agitoxin 2 while bound to the solid support, said substituting of residues being within the 36 amino acid domain defined by -25 to +5 of the selectivity filter where the 0 residue is either the phenylalanine or the tyrosine of the filter's signature sequence selected from the group consisting of 20 glycine-phenylalanine-glycine or glycine-tyrosine-glycine.

In a particular embodiment of the method for screening for compounds as described above, a prokaryote two-transmembrane-domain-type ion channel protein is used, such as 25 from *Steptomyces lividans* especially, the KcsA channel. The channels can be either wild-type or mutated from a wild-type protein. One mutation is confined to the 36 amino acid domain defined by -25 to +5 of the selectivity filter where the 0 residue is either the phenylalanine or the tyrosine of the filter's signature sequence selected from the group consisting of glycine-phenylalanine-glycine or glycine-tyrosine-glycine. The method of this invention includes the use of channel mutations where the protein alteration involves the 30 deletion of a subsequence of the native amino acid sequence and replacement of that native sequence with a subsequence from the corresponding domain of a second and different ion channel protein. The second ion channel protein can be from either a prokaryote or an eukaryote cell.

The methods described above may be conducted using an aqueous solution comprises a nonionic detergent.

In addition to the methods of this invention, the invention further comprises a column having the channel proteins of this invention bound thereto. The proteins are as described
5 herein.

The invention also provides for a non-natural and functional two-transmembrane-domain-type potassium ion channel protein wherein the non-natural protein is mutated in its amino acid sequence from a corresponding natural protein whereby the mutation does not prevent the non-natural protein from binding agitoxin2 when the
10 non-natural protein is further mutated to correspond to the agitoxin2 docking site of a Shaker K⁺ channel protein said docking site created by substituting amino acid residues selected from within the 36 amino acid domain defined by -25 to +5 of the Shaker K⁺ selectivity filter where the 0 residue is either the phenylalanine or the tyrosine of the filter's signature sequence selected from the group consisting of glycine-phenylalanine-glycine or
15 glycine-tyrosine-glycine. It is preferred that the non-natural protein so modified will binds to a channel blocking protein toxin with at least a 10 fold increase in affinity over the native ion channel. The non-natural proteins include those mutations described above for use on a solid support to identify modulators of potassium ion function.

The invention further provides for a means to assess the adequacy of the structural
20 conformation of a two-transmembrane-domain-type potassium ion channel protein for high through put assays comprising the steps of: (i) complexing a two-transmembrane-domain-type potassium ion channel protein having a tetrameric form to a non-lipid solid support under aqueous conditions; (ii) contacting the complexed two-transmembrane-domain-type potassium ion channel protein with a substance known to bind to the two-transmembrane-domain-type potassium ion channel protein when bound to
25 lipid membrane wherein the substance also modulates potassium ion flow in that channel protein; and, (iii) detecting the binding of the substance to the complexed two-transmembrane-domain-type potassium ion channel protein. The channel proteins can be wildtype proteins or modified as described above. Optionally the contacting is done in
30 the presence of a non-ionic detergent and the substance for binding is either a channel blocker or other modulator including a toxin.

What's more, the present invention extends to columns having applications in the methods of the invention. In particular, the present invention extends to a column comprising a solid support having bound thereto an ion channel having the scaffold of a two-transmembrane-domain-type potassium ion channel and having a tetrameric confirmation.

Furthermore, the present invention extends to a column as described above, wherein the ion channel is a non-natural and functional two-transmembrane-domain-type potassium ion channel protein wherein the non-natural protein is mutated in its amino acid sequence from a corresponding natural protein. Such a mutation does not prevent the non-natural protein from binding a toxin, such as agitoxin2 when the non-natural protein is further mutated to correspond to the agitoxin2 docking site of a Shaker K⁺ channel protein. Numerous means are available to the skilled artisan to create the docking. A particular means to create the docking site comprises substituting amino acid residues selected from within the 36 amino acid domain defined by -25 to +5 of the Shaker K⁺ selectivity filter where the 0 residue is either the phenylalanine or the tyrosine of the filter's signature sequence selected from the group consisting of glycine-phenylalanine-glycine or glycine-tyrosine-glycine.

Accordingly, it is a principal object of the present invention to provide a crystal comprising a cation channel protein.

It is another object of the present invention to provide a method for growing a crystal comprising a cation channel protein.

It is yet another object of the present invention to utilize information on the structure of a cation channel protein obtained from a crystal of the present invention, in an assay system for screening potential drugs or agents that may interact with a cation channel protein. Interaction of the potential drug or agent with a cation channel protein includes binding to a cation channel protein, or modulating the function of a cation channel protein, wherein modulation involves increasing the function of a cation channel protein to allow more specific cations to cross a cell membrane, or decrease the function of a cation channel protein to limit or prevent specific cations from permeating through the protein and crossing the cell membrane. Such drugs or therapeutic agents may have broad applications in treating a variety of abnormal conditions, such as cardiac arrhythmia, diabetes mellitus,

seizure disorder, asthma or hypertension, to name only a few.

It is yet another object of the present invention to provide mutant form of a cation channel protein, preferably a potassium channel protein from *Streptomyces lividans*, which binds to Agitoxin2, a toxin found in scorpion venom, in a manner very similar to that in which

- 5 eukaryotic potassium channel proteins bind to Agitoxin2. Consequently, a mutant cation channel protein of the present invention mimics a functional eukaryotic potassium channel protein, and can serve as a model therefor in screening potential drugs or agents that may interact with a eukaryotic potassium channel protein.

It is still yet another object of the present invention to provide a method of preparing

- 10 functional cation channel proteins for use in screen systems for assaying potential drugs or therapeutic agents which may have applications in treating conditions related to the function of cation channel proteins *in vivo*.

It is yet another object of the present invention to provide mutated prokaryotic cation channel proteins which mimic eukaryotic cation channel proteins. With these mutated

- 15 prokaryotic cation channel proteins, drugs or other can be screened for potential interaction with cation channel proteins *in vivo*, and hence, potential use as therapeutic agents in treating conditions related to the function of cation channel proteins *in vivo*, such as cardiac arrhythmia, diabetes mellitus, seizure disorder, asthma or hypertension, to name only a few.

- 20 These and other aspects of the present invention will be better appreciated by reference to the following drawings and Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. (A) Sequence alignment of selected K⁺ channels and cyclic nucleotide-gated channels. The numbering and secondary structural elements for the *Streptomyces lividans* K⁺ channel (kcsa) is given above the sequences. Selectivity filter, red; lining of the cavity and inner pore, blue; residues in which the nature of the side chain is preserved (> 50% similarity), grey. The sequences are: kcsa, *Streptomyces lividans* accession number (acc) 2127577 (SEQ ID NO:1); kch, *Escherichia coli* acc 902457 (SEQ ID NO:2); clost, *Clostridium acetobutylicum* (Genome Therapeutics Corp.) (SEQ ID NO:3); Shaker, *Drosophila melanogaster* acc 85110 (SEQ ID NO:4); hKv1.1, *Homo sapiens* acc 1168947 (SEQ ID NO:5); hDRK, *Homo sapiens* acc 345875 (SEQ ID NO:6); Parame, *Paramecium tetraurelia* acc 643475 (SEQ ID NO:7); *Caenorhabditis elegans* acc 2218158 (SEQ ID NO:8); mSlo, *Mus musculus* acc 539800 (SEQ ID NO:9); cal_act, *Homo sapiens* acc 2832249 (SEQ ID NO:10); AKT1, *Arabidopsis thaliana* acc 2129673 (SEQ ID NO:11); herg, *Homo sapiens* acc 2135973 (SEQ ID NO:12); romk, *Rattus norvegicus* acc 547736 (SEQ ID NO:13); hgirk, *Homo sapiens* acc 1042217 (SEQ ID NO:14); olCNG, *Homo sapiens* acc 2493743 (SEQ ID NO:18); rodCNG, *Homo sapiens* acc 539557 (SEQ ID NO:19). The last two sequences, separate from the rest, are from cyclic nucleotide-gated channels, which are not K⁺ selective.

Fig. 2. Experimental electron density map. Stereo views of the experimental electron-density map contoured at 1 σ covering nearly an entire subunit (removed from the tetramer) of the final model. The map was calculated at 3.2 Å resolution with the following Fourier coefficients: native-sharpened amplitudes and MIR solvent flattened averaged phases. (A) Foreground: map showing inner helix, loop structures and selectivity filter; background: the pore helix and outer helix. CPK spheres show positions of mercury atoms used as residue markers (from the top, marked residues are Leu86, Leu90 and Val93). (B) Alternative view. Foreground: pore helix and part of outer helix; background: selectivity filter and turret. CPK sphere marks position of Ala42. (C) Close up view of electron density.

Fig. 3. Views of the tetramer. (A) Stereo view of ribbon representation illustrating the three-dimensional fold of the kcsa tetramer viewed from the extracellular side. The four subunits are distinguished by color. (B) Stereo view from another perspective,

perpendicular to that in (A). (C) Ribbon representation of the tetramer as an integral-membrane protein. Aromatic amino acids present on the membrane-facing surface are displayed in black. (D) Inverted tepee architecture of the tetramer. These diagrams were prepared with MOLSCRIPT and RASTER-3D (33 of Example I).

- 5 **Fig. 4. Mutagenesis studies on *Shaker*: Mapping onto the kcsa structure.**
Mutations in the voltage-gated *Shaker* K⁺ channel that affect function are mapped to the equivalent positions in kcsa based on the sequence alignment. Two subunits of kcsa are shown. Mutation of any of the white side chains significantly alters the affinity of agitoxin2 or charybdotoxin for the *Shaker* K⁺ channel (12 of Example I). Changing the
10 yellow side chain affects both agitoxin2 and tetraethylammonium ion (TEA) binding from the extracellular solution (14 of Example I). This residue is the external TEA site. The mustard-colored side chain at the base of the selectivity filter affects TEA binding from the intracellular solution (the internal TEA site (15 of Example I)). The side chains colored green, when mutated to cysteine, are modified by cysteine-reactive agents whether or not
15 the channel gate is open, whereas those colored pink react only when the channel is open (16 of Example I). Finally, the residues colored red (GYG, main chain only) are absolutely required for K⁺ selectivity (4 of Example I). This figure was prepared with MOLSCRIPT and RASTER-3D.

- Fig. 5. Molecular surface of kcsa and contour of the pore. (A) A cutaway Stereo view
20 displaying the solvent-accessible surface of the K⁺ channel colored according to physical properties. Electrostatic potential was calculated with the program GRASP, assuming an ionic strength equivalent to 150 mM KCl and dielectric constants of 2 and 80 for protein and solvent, respectively. Side chains of lysine, arginine, glutamate and aspartate residues were assigned single positive or negative charges as appropriate, and the surface coloration
25 varies smoothly from blue in areas of high positive charge through white to red in negatively charged regions. The yellow areas of the surface are colored according to carbon atoms of the hydrophobic (or partly so) side chains of several semi-conserved residues in the inner vestibule (Thr75, Ile100, Phe103, Thr107, Ala108, Ala111, Val115). The green CPK spheres represent potassium ion positions in the conduction pathway. (B)
30 Stereo view of the internal pore running the length of the ion channel. Within a stick model of the channel structure is a three dimensional representation of the minimum radial distance from the center of the channel pore to the nearest van der Waals protein contact.

The display was created with the program HOLE (34 of Example I).

Fig. 6. Identification of permeant ion positions in the pore. (A) A Rb⁺ difference Fourier map calculated to 4.0 Å and contoured at 6 σ identify two strong peaks corresponding to ions in the selectivity filter (inner and outer ions) and a weaker peak 5 corresponding to ions in the cavity (cavity ion). The inner ion density has two closely-spaced peaks. (B) A Cs⁺ difference Fourier map calculated to 5.0 Å and contoured at 6 σ shows the inner and outer ion peaks in the selectivity filter. Both difference Fourier maps were calculated with Fourier coefficients: F(soak) - F(native-unsharpened) and MIR phases. (C) Electron density map contoured at 1 (10 showing diffuse density at the cavity ion position. This map was calculated with the following Fourier coefficients: unsharpened native amplitudes and MIR solvent flattened phases (no averaging information was included).

Fig. 7. Two mechanisms by which the K⁺ channel stabilizes a cation in the middle of the membrane. First, a large aqueous cavity stabilizes an ion (green) in the otherwise 15 hydrophobic membrane interior. Second, oriented helices point their partial negative charge (carboxyl end, red) towards the cavity where a cation is located.

Fig. 8. Detailed views of the K⁺ channel selectivity filter. (A) Stereo view of the experimental electron-density (green) in the selectivity filter. The map was calculated with native-sharpened amplitudes and MIR-solvent-flattened-averaged phases. The selectivity 20 filter of three subunits is shown as a stick representation with several signature sequence residues labeled. The Rb⁺ difference map (yellow) is also shown. (B) Stereo view of the selectivity filter in a similar orientation to (A) with the chain closest to the viewer removed. The three chains represented are comprised of the signature sequence amino acids Thr, Val, Gly, Tyr, Gly (SEQ ID NO:15) running from bottom to top, as labeled in single letter 25 code. The Val and Tyr side chains are directed away from the ion conduction pathway, which is lined by the main chain carbonyl oxygen atoms. Two K⁺ ions (green) are located at opposite ends of the selectivity filter, roughly 7.5 Å apart, with a single water molecule (red) in between. The inner ion is depicted as in rapid equilibrium between adjacent coordination sites. The filter is surrounded by inner and pore helices (white). Although not 30 shown, the model accounts for hydrogen bonding of all amide nitrogen atoms in the selectivity filter except for that of Gly77. (C) A section of the model perpendicular to the

pore at the level of the selectivity filter and viewed from the cytoplasm. The view highlights the network of aromatic amino acids surrounding the selectivity filter. Tyrosine 78 from the selectivity filter (Y78) interacts through hydrogen bonding and van der Waals contacts with two Trp residues (W67, W68) from the pore helix.

5 **Fig. 9.** Sequence alignment of residues 51 to 86 of kcsa K⁺ (SEQ. ID NO:1) and *Shaker* K⁺ (SEQ. ID NO:4) channel pore regions. The numbering for kcsa is given above the sequences. Structural elements are indicated (5 of Example II). Asterisks mark several *Shaker* K⁺ channel amino acid locations where mutations influence Agitoxin2 binding (4, 8, 9 of Example II). Arrows mark the three kcsa K⁺ channel amino acids mutated in this
10 study. The sequences are: kcsa, *Streptomyces lividans* accession number (acc) 2127577 and Shaker, *Drosophila melanogaster* acc 85110.

Fig. 10. Mass Spectra of scorpion toxins before and after channel column purification.
MALDI-TOF mass spectra of venom before purification (A) and after elution from a cobalt column in the absence (B) and presence (C) of attached mutant kcsa K⁺ channel. The
15 accuracy of the mass measurements (\pm 0.3 Da) permitted identification of most of the major peaks in the mass spectra searched from databases of known toxins of the *Leiurus quinquestriatus hebraeus* scorpion (D). The kcsa-binding component labeled * could not be assigned to a known scorpion toxin. The component labeled X (4193.0 Da) binds nonspecifically to the column and was not identified. MALDI_MS was performed with the
20 MALDI matrix 4-hydroxy- α -cyano-cinnamic acid (16 of Example II).

Fig. 11. Binding affinity of wild type and mutant Agitoxin2 to the mutant kcsa K⁺ channel. (A) Quantity of radiolabeled Agitoxin2 bound to 0.3 μ l of cobalt resin saturated with the mutant kcsa K⁺ channel is shown as a function of the radiolabeled Agitoxin2 concentration (17 of Example II). Each point is the mean \pm SEM of 4 measurements,
25 except for the 0.03 μ M and 1.5 μ M concentrations which are the mean \pm range of mean of two measurements. The curve corresponds to equation Bound Agitoxin2 = A * {1 + K_d / [Agitoxin2]}⁻¹, with equilibrium dissociation constant K_d = 0.62 μ M and resin capacity A = 16 pMoles. (B) Remaining bound fraction of radiolabeled wild type toxin is graphed as a function of the concentration of unlabeled wild type toxin or mutant toxins K27A or
30 N30A (17). Each point is mean \pm SEM of 4 measurements for wild type Agitoxin2 (squares) or mean \pm range of mean of 2 measurements for K27A (circles) and N30A

(triangles) Agitoxin2 mutants. The curves correspond to equation Remaining Bound Fraction = {1 + Kdhot / [Thot]} * {1 + (Kdhot / [Thot]) * (1 + [Tcold] / Kdcold)}⁻¹ with labeled toxin concentration Thot = 0.06 μM, wild type toxin Kdhot = 0.62 μM, and competing toxin dissociation constant Kdcold = 0.62 μM (wild type), 81 μM (K27A), and 5 27 μM (N30A). (C) CPK model of Agitoxin2 viewing the interaction surface. Side chains of functionally important amino acids are shown in red (4 of Example II). This figure was prepared using the program GRASP (19 of Example II).

Fig. 12. Docking of Agitoxin2 onto the kcsa K⁺ channel. (A) Molecular surface of the pore entryway of the kcsa K⁺ channel (left) and Agitoxin2 (right). The colors indicate 10 locations of interacting residues on the toxin and channel surfaces as determined by thermodynamic mutant cycle analysis of the *Shaker* K⁺ channel-Agitoxin2 interaction (4,8 of Example II). The three pore mutations of the kcsa K⁺ channel used in this study (Q58A, T61S, R64D) were introduced into the channel model using the program O (19 of Example II). Indicated residues on the channel surface correspond to the positions of the *Shaker* K⁺ 15 channel equivalent residues (See Fig. 9) which couple to the indicated Agitoxin2 residues. (B) The pattern of colors in (A) suggests the docking orientation shown by the main worm representation of Agitoxin2 placed manually onto the pore entryway. The side chain colors match the colored patches in (A). Gly10 is shown as a green band on the worm. The mutant cycle coupling between residues at *Shaker* 425 (mutant kcsa 58) and residue 10 of 20 Agitoxin2 comes about through substitution of a bulky side chain residue at either position (4, 7 of Example II). Pictures were made using the program GRASP (19 of Example II).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery of a crystal of a cation channel protein, in particular a potassium channel protein from *Streptomyces lividans*, and a method of 25 forming such crystals. Moreover, the present invention is based on the recognition that, based on the structure of the crystalline cation channel protein, potential drugs and therapeutic agents which can bind to cation channel protein can be screened for their use in treating conditions related to the function of cation channel proteins, particularly potassium channel proteins, *in vivo*.

Furthermore, the present invention is based upon the discovery that cation channel proteins from prokaryotic organisms, such as a potassium channel protein from *Streptomyces lividans*, have much similarity and conservation with eukaryotic potassium channel proteins. In particular, a mutated prokaryotic potassium channel protein binds to a 5 particular scorpion toxin in much the same way a eukaryotic potassium channel protein binds to the same toxin.

For purposes of this Application, the term "positioned to extend into the lipid bilayer membrane proximate to the interface..." indicates that aromatic side groups of amino acid residues interject into the lipid bilayer membrane from about 0 Å to about 5 Å from the 10 interface of the lipid bilayer with either the extracellular matrix or the cellular cytosol, i.e., the point at which the lipid bilayer membrane meets either the extracellular matrix or the cellular cytosol.

Moreover, for purposes of this Application, the term "kink" indicates the inner transmembrane alpha-helix comprises a slight bend in its structure. Moreover, the angle of 15 the tilt of the inner transmembrane helix "normal of the lipid bilayer" indicates the amount of tilt in the inner membrane helix relative to a line perpendicular to the lipid bilayer membrane at a point at which the inner transmembrane alpha-helix would have intersected the lipid bilayer membrane, had the inner transmembrane alpha-helix extended thereto.

Moreover, for purposes of this Application the "specific ion" refers to the ion species 20 intended to permeate a particular cation channel protein. For example, if the K⁺ is the specific ion for a potassium channel protein, Na⁺ is the specific ion for a sodium channel protein, and Ca²⁺ is the specific ion for calcium channel protein.

Furthermore, an α-helix in a protein is found when a stretch of consecutive residues all have a phi,psi angle pair of approximately -60° and -50°, corresponding to the allowed 25 region of a Ramachandran plot (Branden, C. And Tooze, J. Introduction to Protein Structure, Garland Publishing, Inc. New York and London, 1991 p.12 (this reference is incorporated by reference herein in its entirety)).

Moreover, the term "bundle" of α-helices, as used herein, refers to the packing at least two α-helices closely together by intercalating side chains of residues of the helices in the

interation area among them.

The term "stack of sequential oxygen rings" as used herein refers to oxygen atoms of side chains of amino acid residues, such as carbonyl groups, lining a selectivity filter of a 5 cation channel protein which interact with the specific ion in order to permit it to enter the central pore of a cation channel protein, and cross a lipid bilayer membrane.

Further, the term "abut" as used herein indicates an α -helix is adjacent to an the lipid bilayer of a lipid bilayer membrane.

The term describing a protein found "natively in a lipid bilayer membrane" refers to a 10 membrane bound protein, such as a cation channel protein which is in its biologically active conformation, and located in the cellular lipid bilayer membrane.

Furthermore, the term "communicates" refers to connections between individual parts of a central pore of a cation channel protein so that the specific cation is can pass through all 15 individual parts of the central pore, and cross a cellular lipid bilayer membrane via the cation channel protein.

Moreover, the term "agent" as used throughout the instant Application refers to any potential ligand of a cation channel protein, wherein such potential ligands include, but are not limited to, small molecules, both synthetic and naturally occurring, biodegradable cofactors, proteins, synthetic peptides, or polymers, both synthetic and naturally 20 occurring, including DNA.

As used herein, the term "Agitoxin 2" refers to a neurotoxin from *Leiurus Quinquestriatus Hebraeus* which is a scorpion. The amino acid sequence has been identified and the gene has been cloned and expressed. The amino acid sequence is known and available under Accession No. 1065324 in the GenPept Data Base located at 25 WWW.NCBI.NLM.NIH.GOV on the World Wide Web.

Also, as used herein, the phrase "Agitoxin 2 docking site" refers to the amino acids which

physically interact with Agitoxin 2 and are primarily responsible for conferring the ability of a channel protein to bind to Agitoxin 2.

As used herein, the term "functional" refers to a channel protein which is in a tetrameric form and having a confirmation that is sufficiently reflective of the native protein in its 5 natural environment so that when a compound binds to the functional channel protein that same compound would also bind to that protein in its natural environment. The test for determining if a channel protein is functional is provided below and relies upon the ability of the protein to bind Agitoxin 2 when deliberately mutated to bind the toxin.

"Non-natural" refers to a potassium ion channel protein that has been modified or altered 10 from a corresponding wild type protein. Typically the protein is altered in its primary amino acid sequence but fusions and chimera to the N and C terminus are included as well as addition of non-protein components to available reactive sites.

As used herein, "natural" refers to a potassium ion channel protein which is found in nature. This is referred to as a wildtype.

15 The term "mutated" as used herein refers to a potassium ion channel protein that has been altered by deletion, substitution or addition of amino acids.

As used herein, the phrase "selectivity filter" refers to the domain of channel ion protein that is responsible for the ability of the protein to exclude one or a group of ions and to allow other ions to pass.

20 As used herein, the phrase "signature sequence" refers to a sequence of amino acids which define the protein as that protein or as belonging to a group or family of proteins. For specific proteins the signature sequence may be very conserved and be a unique identifier. For signature sequences that define a family, the sequence would be relatively hypervariable but conserved across the family.

25 Also, as used herein, "solid supports" refer to any non-soluble matrix upon which the potassium ion channel proteins of this invention may be attached.

As used herein, the phrase "structural conformation" refers to a physical relationship between amino acids within a protein. It is a relative state which alters with salt concentration, temperature and hydrophobic nature of the solvent being used. Structural confirmation is best defined by function.

- 5 The phrase "tetrameric protein" used herein refers to a protein having quaternary structure comprising 4 subunits which may be the same or different.

As used herein, the phrase "two-transmembrane-domain type potassium ion channel protein" refers to potassium channel monomer having two regions of hydrophobicity with sufficient length to form transmembrane segments. Between these two segments must be
10 found the potassium channel signature sequence. When using the tyrosine or phenylalanine residue of the signature sequence as a zero reference point, the first transmembrane segment would begin within approximately -61 residues of the reference point and the second transmembrane would end within approximately +42 amino acids of the reference point. To identify the two transmembrane domains one can construct a Kyte-Dolittle
15 hydropathy plot of the amino acids.

As used herein, the phrase "wild-type" protein refers to a protein such as a potassium ion channel protein which is presented with a primary amino acid sequence that is found in nature.

Isolation of a functional cation channel protein for use in assays to screen potential drugs
20 and therapeutic agents.

This method of the present invention overcomes limitations of using cation channel proteins in the development of drugs or therapeutic agents to treat conditions related to the function of cation channel proteins, and particularly potassium cation channel proteins *in vivo*, such as cardiac arrhythmia, diabetes mellitus, seizure disorder, asthma or hypertension, to name
25 only a few.

In particular, since cells need very few potassium channels in order to function, it is difficult to isolate functional potassium channels in great quantities. Moreover, recombinant techniques to have a cell produce excess potassium channel proteins has met with only

limited success. As a result, very few drugs or agents are currently available which act on potassium channel proteins.

However, Applicant has discovered a method to isolate cation channel proteins, particularly potassium cation channel proteins, which can then be used in efficient assays to screen potential drugs and agents for interaction with such proteins. In particular, disclosed herein is a method of using a functional cation channel protein in an assay for screening for potential drugs or agents that may bind to a cation channel protein comprising, wherein the assay comprises the steps of providing a functional cation channel protein, conjugating the functional cation channel protein to a solid phase resin, contacting the potential drug or agent to the functional cation channel protein conjugated to the solid phase resin, removing the functional cation channel protein from the solid phase resin, and determining whether the potential drug or agent is bound to the cation channel protein.

Since cation channel proteins are trans membrane bound proteins, care should be taken in their isolation. In particular, to prevent denaturation and a loss of functional activity, they require a hydrophobic environment. In a preferred embodiment, a functional cation channel protein is provided by expressing an isolated nucleic acid molecule encoding the cation channel protein in a unicellular host such that the cation channel protein is present in the cell membrane of the unicellular host, lysing the unicellular host in a solubilizing solution so that the cation channel protein is solubilized in the solution, and extracting the cation channel protein from the solubilizing solution with a detergent.

Many solubilizing solutions are presently known to one of ordinary skill in art, which can solubilize a cation channel protein, and prevent its denaturation or proteolytic digestion. All such solutions are encompassed by the present invention. In a preferred embodiment, the solubilizing solution comprises Tris buffer, 100 mM KCl, 10 mM MgSO₄, 25 mg DNase 1, 250 mM sucrose, pepstatin, leupeptin, and PMSF at pH 7.5.

Moreover, many detergents are available to the skilled artisan for extracting solubilized cation channel protein from a solubilizing solution of the present invention. Examples of detergents having applications herein include SDS, Triton 100, glycerol, decylmaltoside, Tween-20, or Tween-80, to name only a few. In a preferred embodiment, a 40 mM decylmaltoside is used to extract the cation channel protein from a solubilizing solution of

the present invention.

Furthermore, Applicant has discovered that cation channel proteins, particularly potassium cation channel proteins, can be conjugated chemically to a solid phase resin. As a result, the channel proteins are immobilized and readily available in assays for screening drugs or agents that may bind to a cation channel protein. In a preferred embodiment, a cation channel protein is conjugated to a cobalt resin through a carboxyl terminal hexahistidine tag.

In preferred embodiment, cation channel proteins are conjugated to a cobalt resin at a protein to resin ratio that allows for saturation of the resin with the cation channel protein. 10 As a result, numerous cation channel proteins are immobilized and available for contact with a potential drug or therapeutic agent to be screened pursuant to the present invention.

Moreover, numerous screening methods are available and encompassed by the present invention. For example, the resin with the cation channel conjugated thereto can be incubated in a solution comprising the potential drug or therapeutic agent. In another 15 embodiment, the resin can be used to line a column, to which the potential drug or agent is added. Preferably, a potassium ion channel protein from *Streptomyces lividans* comprising an amino acid sequence of SEQ ID NO:1, or conserved variants thereof, is mutated to mimic a eukaryotic potassium channel, such as a potassium channel protein of *Drosophila melanogaster* comprising an amino acid sequence of SEQ ID NO:4, or conserved variants. 20 Consequently, the mutated potassium channel protein of *Streptomyces lividans* comprising an amino acid sequence of SEQ ID NO:16 is conjugated to a cobalt resin, which is then used to line a 1 ml column. A composition comprising the potential drug or agent to be screened for interaction with a eukaryotic cation channel protein is then poured into the 25 column, so that the potential drug or agent can contact the mutated prokaryotic cation channel protein conjugated to the cobalt membrane.

After contact, the cation channel proteins are removed from the resin, and examined for interaction binding with the potential drug or agent. Numerous methods of cleaving a protein from a solid phase resin are available to the skilled artisan, and included in the present invention. In a preferred embodiment, the removing step comprises contacting the 30 cation channel protein conjugated to the resin to an imidazole solution. The cation channel

proteins can then be collected, and examined for interaction, i.e. binding, with the potential drug or therapeutic agent.

Furthermore, determining whether the drug or therapeutic agent is bound to the cation channel protein can be done with numerous methods. For example, molecular weight determinations can be made with SDS-PAGE comparing the molecular weight of the cation channel protein not contacted with the drug, to the molecular weight of the cation channel protein contacted with the drug. Furthermore, other analytical methods, such as HPLC, mass spectrometry, or spectrophotometry, to name only a few, can be used to determine whether the drug or agent is bound to a cation channel protein previously conjugated to a solid phase resin.

Moreover, screening potential drugs or agents which may bind a cation channel protein may be performed on an individual basis, i.e. one potential drug or agent at a time, or the present invention can be used to screen whole libraries of compounds at one time, such as a mixture of compounds or a combinatorial library, for potential drugs or agents which potentially bind to a cation channel protein. For example, combinatorial libraries which can be screened with the present invention include, but are not limited to, a phage display library, in which numerous proteins and polypeptides are being express simultaneously, libraries comprising synthetic peptides.

Two-transmembrane-domain type potassium ion channel proteins

As set forth above, two-transmembrane type potassium ion channel proteins are well known and structurally constitute one of the classes of potassium channels. They are found in a wide variety of organisms, both prokaryotic and eukaryotic where they serve the purpose of controlling the influx or efflux of potassium ions across cell membranes. Potassium channels as a class are tetrameric membrane proteins characterized by multiple transmembrane segments and a pore region through which potassium ions flow. These channels may be homotetrameric, that is, consisting of four identical monomers, or heterotetrameric, consisting of four monomers which are not necessarily identical. The individual monomers of the heterotetrameric forms are usually structurally related, and may or may not form a functional potassium channel when reconstituted as homotetramers of themselves. The pore region contains a signature sequence consisting of

glycine-tyrosine-glycine or glycine-phenylalanine-glycine. Each monomer in the tetrameric structure contributes to the formation of the pore region, and each subunit contains a signature sequence.

- To identify a putative protein as a two-transmembrane potassium channel monomer,^a
- 5 Kyte-Dolittle hydropathy plot of the amino acid may be constructed, and it should demonstrate two regions of hydrophobicity with sufficient length to form transmembrane segments. Between these segments must be found the potassium channel signature sequence. When using the tyrosine or phenylalanine residue of the signature sequence as a zero reference point, the first transmembrane segment would begin within approximately
- 10 -61 residues of the reference point and the second transmembrane would end within approximately +42 amino acids of the reference point.

- Potassium channel monomer subunits may be obtained by a variety of methods, including cloning by nucleic acid hybridization, cloning by antibody selection of expressed proteins, and using the polymerase chain reaction (PCR) with homologous or degenerate primer sets.
- 15 One of skill in the art would be able to readily obtain DNA sequence encoding such potassium channels given a known DNA sequence or an antibody against the channel itself.

- Examples of proteins which have been cloned and identified as two-transmembrane potassium ion channels include IRK3 as described in Koyama H, *et al.*, Molecular cloning, functional expression and localization of a novel inward rectifier potassium channel in the rat brain. FEBS Lett 341:303-7 1994; IRK3 as described in Morishige et al., Molecular cloning and functional expression of a novel brain-specific inward rectifier potassium channel. FEBS Lett 346: 251-6, 1994; UKATP reported in Inagaki et al., Cloning and functional characterization of a novel ATP-sensitive potassium channel ubiquitously expressed in rat tissues, including pancreatic islets, pituitary, skeletal muscle, and heart. J Biol Chem 270:5691-4; and GIRK2 reported in Ferrer *et al.*, Pancreatic islet cells express a family of inwardly rectifying K⁺ channel subunits which interact to form G-protein-activated channels. J Biol Chem 270:26086-91 1995.

Mutations of two-transmembrane-domain type potassium ion channel proteins

The present invention further extends to introducing Agitoxin2 docking sites into

- two-transmembrane-domain type potassium ion channel protein. Any two transmembrane cation channel protein presently known, or subsequently discovered, can routinely be modified to bind agitoxin2 using the protocols described *infra*. As explained herein, scorpion toxins, such as agitoxin2, bind to an ion channel by making contact with all four subunits where they
- 5 come together to form the pore. Hence, such toxins will only bind to the channel if the subunits have been properly assembled. As a result, the binding of a toxin, such as agitoxin2, to a non-natural two transmembrane cation channel protein can be used to confirm the template channel integrity or function, i.e., to confirm the two-transmembrane cation channel protein has been properly modified to mimic a functional eukaryotic two-transmembrane cation channel protein.
- 10 The general method for creating an agitoxin (or related scorpion toxin) binding site on the template channel is now described. Particular examples of pore region sequences (toxin binding sequences) of four two-transmembrane cation channel proteins having applications in the present invention are described below:

Shaker aeagsensffksipdafwwavvtmttvgygdmtpvgfwgk

15 Romk1 anhtpcveningltsaflsletqvtigygfrcvteqcat
Mjan esvilmtvegwdfafftaytavvtistvgygdtytpqtflgkls
KcsA vlaerpgaqilitypralwwsvetattvgygdlypvtlwgr

Shaker is a six-membrane spanning K channel from *Drosophila melanogaster*, ROMK1 is a
20 two membrane-spanning K channel from rat renal outer medulla (kidney). Mjan is a two membrane-spanning K channel from *Meihancoccus janschii*, and KcsA is a two membrane-spanning K channel from the bacterium *Streptomyces lividans*.

As explained herein, cation channel proteins have a high degree of sequence conservation, particularly in the region of the selectivity filter. Hence, ggg sequence should be used as a
25 reference to align the sequences. The underlined amino acids on the Shaker channel sequence are known to be important for binding of agitoxin, as described *infra*. In particular, described herein is the mutating of several of the underlined amino acids, using standard techniques. As a result of these mutations, the KcsA K channel became sensitive to agitoxin binding. Similarly, other channels can be subjected to the same analysis. Therefore, using the teachings set forth

infra, Mjan or Romk1 channels can readily be modified by those of ordinary skill. Numerous techniques are readily available to the skilled artisan to convert the appropriate (underlined) amino acids of the pore regions of the two-transmembrane cation channel proteins described above to the amino acid residues found in the corresponding position of the Shaker K channel.

- 5 A particular technique which can be used in this modification process is directed mutagenesis.

Also, the present invention involves introducing mutations into the two-transmembrane-domain type potassium ion channel protein which allow it to mimic other potassium ion channel proteins. In particular, the present invention contemplates the use of two-transmembrane proteins as a scaffold for studying or identifying modulators of 10 potassium ion channel function. The proteins can be modified in a variety of different ways to mimic or simulate properties of related potassium ion channels including conferring properties found in six membrane domain type ion channels. Accordingly, one can create channel proteins that have been minimally altered from their corresponding wild type for convenience of purification, i.e. removing protease cleavage sites in noncritical 15 domains, or attaching binding domains to facilitate chromatographic purifications such as FLAG or polyHis. Because the overall structure of potassium ion proteins is conserved, modifications can be introduced that can transfer properties of one channel protein to the two-transmembrane proteins that is being used as a scaffold. Among these modifications are venom docking sites as exemplified herein as well as binding sites for modulators such 20 as to the transmembrane domains and alterations to the ion filter region.

Recombinant genetics has a variety of techniques for introducing and for determining the domains and in many cases the specific amino acids which are responsible for the physical properties of channel proteins. In brief, these methods consist of manipulating the amino acid sequence of a protein in order to identify which part of the protein is involved in the 25 structure or function of the molecule and then transferring that domain and its properties to proteins that do not naturally have that property. These methods have already been widely applied in the study of ion channels. The study of ion channels lends itself very well to such methods, because these proteins exist in a number of functional families within which are numerous structurally related yet biophysically and pharmacologically distinct 30 subfamily members. For example, the superfamily of potassium channels all share the pore signature sequence gly-tyr-gly or gly-phe-gly, and are tetrameric; subfamily monomers may have two transmembrane segments or 6 transmembrane segments, and may be gated

by membrane potential, intracellular calcium concentration, intracellular cyclic nucleotides, membrane deformation, and pH; they may be inwardly rectifying, outwardly rectifying, or nonrectifying; and their activation and inactivation kinetics, and conductances may vary tremendously.

- 5 As exemplified in this application, a number of scorpion and bee venom toxins can bind with high affinity to one subfamily member while being inactive on a closely related subfamily members. It is therefore not surprising that amino acid sequence mutations which confer the properties of one ion channel upon another are a tool which has been commonly employed by ion channel researchers and this invention takes advantage of this
- 10 pleiomorphic property in the super family of potassium channels.

- Mutations may be introduced using a number of approaches, each with its own particular strengths. Often a combination of these may be used to generate a channel with altered properties. Examples of these approaches are deletions of amino acids, domain replacement of one channel with that of a different channel (chimeras), replacement of
- 15 amino acids with different amino acid in a nontargeted or semi-targeted way (e.g. alanine-scanning mutagenesis) and replacement of targeted amino acids with different amino acids (site-directed mutagenesis). Although each method may be applied independently, oftentimes several or all of these may be employed to arrive at a mutant channel with the desired characteristics. Examples of changed characteristics include channel gating,
 - 20 voltage response, rectification, ion preference, and the binding of small organic molecules and peptides to the channel.

- Mutagenesis is especially powerful when an ion channel with novel toxin or small organic molecule-binding characteristics is required. Using this approach, channels which do not show significant binding of a particular toxin or small organic molecule may be engineered
- 25 to bind strongly to these molecules. Conversely, channels which strongly bind a particular toxin or small organic molecule may be engineered to lose that property.

- Examples of the use of the chimeric and site-directed approach are many. In Ishii, T.M., Maylie, J. and Adelman, J.P. (1997) J. Biol. Chem 272: 23195-200, the authors were able to confer apamin sensitivity on a channel which did not possess this property. Similar
- 30 studies have been performed on the Kv1.3 and Kv2.1 potassium channels by Gross et al.

(1994), Neuron 13: 961-6. In their study, they transferred scorpion toxin sensitivity from the highly sensitive Kv1.3 potassium channel to the insensitive Kv2.1 potassium channel by transferring the stretch of amino acids between transmembrane domains 5 and 6.

Conversely, alanine-scanning mutagenesis was used by Hanner et al. (1998), J Biol Chem 5 273: 16289-96, to impair charybdotoxin binding to the maxi-K channel, and direct point mutations were employed by Wang and Wang (1998), Proc Natl Acad Sci U S A 95:2653-8, to remove batrachotoxin sensitivity from sodium channels.

Mutagenesis may also be employed to alter the biophysical properties of ion channels, in effect causing one channel to have characteristics similar to those of another. For example,

10 voltage-gated potassium channels of the *Shaker* subfamily open in response to changes in membrane potential. Members of this subfamily of potassium channels have the intrinsic property of opening at different membrane potentials depending on the particular family member, and have the characteristic of delayed rectification. Liman et al., (1991), Nature, 353:752-6, were able to demonstrate that mutations in the S4 voltage sensor domain of

15 *Shaker* changed the opening potential; by mutating several amino acid residues in the S4 voltage sensor domain of *Shaker*, Miller and Aldrich (1996), Neuron, 16:853-8, were able to convert this channel from a delayed rectifier into a voltage-gated inward rectifier. Chimeric constructs may use related domains from different channel types. The rat CNG olfactory channel is a member of the voltage-gated subfamily of potassium channels, but is

20 itself voltage-independent and is not entirely selective for potassium ions as compared with the eag channel. Tang and Papazian (1997), J Gen Physiol, 109:301-11, were able to convert the human eag potassium channel from a voltage sensitive to a voltage-independent channel by substituting the S3-S4 domain of the rat cyclic-nucleotide gated (CNG) olfactory channel.

25 It is therefore clear that mutagenesis may be readily used to confer the pharmacological and biophysical properties of one channel upon another, and that this methodology applies to not only potassium, but sodium and calcium channels.

Determining if the two-transmembrane-domain type potassium ion channel protein has maintained function using Agitoxin2 binding. Beyond the ability of the channel proteins of

30 this invention to pass ions under *ex vivo* conditions or using liposomes, their functionality can measured by the ability to be modified to accept or recognize agitoxin2. To

accomplish this one follows the mutagenesis methods described above both generically for mutation of any channel protein and for the introduction of an agitoxin2 docking site into any two transmembrane-type domain potassium ion channel protein.

- Once mutated, the proteins are tested by any number of binding assay formats including
- 5 homogenous assays where both agitoxin2 and the channel protein are free in solution and heterogeneous assay formats where one of the binding members is bound to a solid support. Either member can be labelled using the labels described herein. The preferred method for assaying for agitoxin2 binding uses the cobalt resin and procedures described in Example II.

10 Binding the two-transmembrane-domain type potassium ion channel protein to solid supports.

The potassium channels of the invention can be bound to a variety of solid supports. Solid supports of this invention include membranes (e.g., nitrocellulose or nylon), a microtiter dish (e.g., PVC, polypropylene, or polystyrene), a test tube (glass or plastic), a dip stick 15 (e.g., glass, PVC, polypropylene, polystyrene, latex and the like), a microfuge tube, or a glass, silica, plastic, metallic or polymer bead or other substrate such as paper. A preferred solid support uses a cobalt or nickel column which binds with specificity to a histidine tag engineered onto the channel proteins.

Adhesion of the channel proteins to the solid support can be direct (i.e. the protein contacts 20 the solid support) or indirect (a particular compound or compounds are bound to the support and the target protein binds to this compound rather than the solid support). One can immobilize channel proteins either covalently (e.g., utilizing single reactive thiol groups of cysteine residues (*see, e.g.*, Collioud *et al.* *Bioconjugate Chem.* 4:528-536 (1993)) or non-covalently but specifically (*e.g.*, via immobilized antibodies (Schuhmann *et 25 al.* *Adv. Mater.* 3:388-391 (1991); Lu *et al.* *Anal. Chem.* 67:83-87 (1995), the biotin/streptavidin system (Iwane *et al.* *Biophys. Biochem. Res. Comm.* 230:76-80 (1997) or metal chelating Langmuir-Blodgett films (Ng *et al.* *Langmuir* 11:4048-55 (1995); Schmitt *et al.* *Angew. Chem. Int. Ed. Engl.* 35:317-20 (1996); Frey *et al.* *Proc. Natl. Acad. Sci. USA* 93:4937-41 (1996); Kubalek *et al.* *J. Struct. Biol.* 113:117-123 (1994)) and 30 metal-chelating self-assembled monolayers (Sigal *et al.* *Anal. Chem.* 68:490-497 (1996))

for binding of polyhistidine fusions.

Indirect binding can be achieved using a variety of linkers which are commercially available. The reactive ends can be any of a variety of functionalities including, but not limited to: amino reacting ends such as N-hydroxysuccinimide (NHS) active esters,

- 5 imidoesters, aldehydes, epoxides, sulfonyl halides, isocyanate, isothiocyanate, and nitroaryl halides; and thiol reacting ends such as pyridyl disulfides, maleimides, thiophthalimides, and active halogens. The heterobifunctional crosslinking reagents have two different reactive ends, e.g., an amino-reactive end and a thiol-reactive end, while homobifunctional reagents have two similar reactive ends, e.g., bismaleimidohexane (BMH) which permits
- 10 the cross-linking of sulfhydryl-containing compounds. The spacer can be of varying length and be aliphatic or aromatic. Examples of commercially available homobifunctional cross-linking reagents include, but are not limited to, the imidoesters such as dimethyl adipimidate dihydrochloride (DMA); dimethyl pimelimidate dihydrochloride (DMP); and dimethyl suberimidate dihydrochloride (DMS).
- 15 Heterobifunctional reagents include commercially available active halogen-NHS active esters coupling agents such as N-succinimidyl bromoacetate and N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB) and the sulfosuccinimidyl derivatives such as sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (sulfo-SIAB) (Pierce). Another group of coupling agents is the heterobifunctional and thiol cleavable agents such as
- 20 N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Pierce).

- Antibodies are also available for binding channel proteins to a solid support. This can be done directly by binding channel protein specific antibodies to the column and allowing channel proteins to bind or it can be done by creating chimeras constructed from the channel protein linked to an appropriate immunoglobulin constant domain sequence. they
- 25 are termed immunoadhesins and they are known in the art. Immunoadhesins reported in the literature include Gascoigne et al., Proc. Natl. Acad. Sci. USA 84, 2936-2940 (1987), Capon et al., Nature 377, 525-531 (1989); and Traunecker et al., Nature 33, 68-70 (1989).

By manipulating the solid support and the mode of attachment of the target molecule to the support, it is possible to control the orientation of the target molecule. Thus, for example,

where it is desirable to attach a target molecule to a surface in a manner that leaves the molecule tail free to interact with other molecules, a tag (e.g., FLAG, myc, GST, polyHis, etc.) may be added to the target molecule at a particular position in the target sequence.

- It is also possible to reconstitute channels in lipid, membranes or liposomes. For
5 example the following references teach how to reconstitute the channel proteins of this invention in membranes. The very channels of this invention, SliK, the K⁺ channel encoded by the Streptomyces KcsA gene, was expressed, purified, and reconstituted in liposomes. See, Heginbotham L *et al.* J Gen Physiol 1998 Jun;111(6):741-9 and in Cuello LG, *et al.*, Biochemistry 1998 Mar 10;37(10):3229-36. In Shin, JH *et al.*, FEBS Lett 1997
10 Oct 6;415(3):299-302 where the authors demonstrated that nitric oxide could activate a calcium-activated potassium channel from rat using the planar lipid bilayer technique.
Santacruz-Toloza L *et al.* Biochemistry 1994 Feb 15;33(6):1295-9.

Assays.

- Once bound there are a variety of assay formats that can be used to screen for modulators
15 of the channel proteins. Various molecules that interact with a potassium channel can be identified by 1) attaching the potassium channel ("the target") to a solid support, 2) contacting a second molecule with the support coated with the potassium channel, and 3) detecting the binding of the second molecule to the potassium channel. Molecules that interact or bind with the target are then eluted, with or without the target, thereby isolating
20 molecules that interact with the target.

- For a general description of different formats for binding assays, see BASIC AND CLINICAL IMMUNOLOGY, 7th Ed. (D. Stiles and A. Terr, ed.)(1991); ENZYME IMMUNOASSAY, E.T. Maggio, ed., CRC Press, Boca Raton, Florida (1980); and "Practice and Theory of Enzyme Immunoassays" in P. Tijssen, LABORATORY
25 TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY, Elsevier Science Publishers, B.V. Amsterdam (1985), each of which is incorporated by reference.

In competitive binding assays, the test compound competes with a second compound for specific binding sites on a target molecule attached to the solid support. Binding is determined by assessing the amount of second compound associated with the target

molecule. The amount of second compound associated with the target molecule is inversely proportional to the ability of a test compound to compete in the binding assay.

The amount of inhibition or stimulation of binding of a labeled target by the test compound depends on the binding assay conditions and on the concentrations of binding agent, labeled analyte and test compounds used. Under specified assay conditions, a compound is said to be capable of inhibiting the binding of a second compound to a target compound if the amount of bound second compound is decreased by 50% or preferably 90% or more compared to a control sample.

Alternatively, various known or unknown compounds, including proteins, carbohydrates, and the like, can be assayed for their ability to bind to the channels of this invention. In one embodiment, samples from various tissues are contacted with the target to isolate molecules that interact with the target. In another embodiment, small molecule libraries and high throughput screening methods are used to identify compounds that bind to the target.

15 Labels for use in assays.

The amount of binding of the second compound to a target channel protein can be assessed by directly labeling the second compound with a detectable moiety, or by detecting the binding of a labeled ligand that specifically binds to the second compound. A wide variety of labels can be used. The detectable labels of the invention can be primary labels (where the label comprises an element that is detected or that produces a directly detectable signal) or secondary labels (where the detected label binds to a primary label, e.g., as is common in immunological labeling). An introduction to labels, labeling procedures and detection of labels is found in Polak and Van Noorden (1997) *Introduction to Immunochemistry*, 2nd ed., Springer Verlag, NY and in Haugland (1996) *Handbook of Fluorescent Probes and Research Chemicals*, a combined catalog and handbook published by Molecular Probes, Inc., Eugene, OR. Useful primary and secondary labels of the present invention can include spectral labels such as fluorescein isothiocyanate (FITC) and Oregon Green™, rhodamine and derivatives (e.g. Texas red, tetrarhodamine isothiocyanate (TRITC), etc.), digoxigenin, biotin, phycoerythrin, AMCA, CyDyes™, and the like), radiolabels (e.g., ³H, ¹²⁵I, ³⁵S, ¹⁴C or ³²P), enzymes (e.g. horseradish peroxidase, alkaline phosphatase, etc.).

spectral colorimetric labels such as colloidal gold and colored glass or plastic (e.g. polystyrene, polypropylene, latex, etc.) beads. The choice of label depends on sensitivity required, ease of conjugation with the compound, stability requirements, and available instrumentation.

- 5 In general, a detector that monitors a particular probe or probe combination is used to detect the recognition reagent label. Typical detectors include spectrophotometers, phototubes and photodiodes, microscopes, scintillation counters, cameras, film and the like, as well as combinations thereof. Examples of suitable detectors are widely available from a variety of commercial sources known to persons of skill.

10 High-Throughput Screening of Candidate Agents that Modulate Potassium Channel Proteins.

- Conventionally, new chemical entities with useful properties are generated by identifying a chemical compound (called a "lead compound") with some desirable property or activity, creating variants of the lead compound, and evaluating the property and activity of those
15 variant compounds. However, the current trend is to shorten the time scale for all aspects of drug discovery. Because of the ability to test large numbers quickly and efficiently, high throughput screening (HTS) methods are replacing conventional lead compound identification methods.

- In one preferred embodiment, high throughput screening methods involve providing a
20 library containing a large number of potential therapeutic compounds (candidate compounds). Such "combinatorial chemical libraries" are then screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or
25 actual therapeutics.

Combinatorial chemical libraries

Combinatorial chemical libraries are a preferred means to assist in the generation of new chemical compound leads. A combinatorial chemical library is a collection of diverse

- chemical compounds generated by either chemical synthesis or biological synthesis by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks called amino acids in every possible way for a given compound length (*i.e.*, the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks. For example, one commentator has observed that the systematic, combinatorial mixing of 100 interchangeable chemical building blocks results in the theoretical synthesis of 100 million tetrameric compounds or 10 billion pentameric compounds (Gallop *et al.* 10 (1994) 37(9): 12331250).

Preparation and screening of combinatorial chemical libraries are well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (*see, e.g.*, U.S. Patent 5,010,175, Furka (1991) *Int. J. Pept. Prot. Res.*, 37: 487-493, Houghton *et al.* (1991) *Nature*, 354: 84-88). Peptide synthesis is by no means the only approach envisioned and intended for use with the present invention. Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (PCT Publication No WO 91/19735, 26 Dec. 1991), encoded peptides (PCT Publication WO 93/20242, 14 Oct. 1993), random biooligomers (PCT Publication WO 92/00091, 9 Jan. 1992), benzodiazepines (U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al.*, (1993) *Proc. Nat. Acad. Sci. USA* 90: 69096913), vinylogous polypeptides (Hagihara *et al.* (1992) *J. Amer. Chem. Soc.* 114: 6568), nonpeptidal peptidomimetics with a Beta D Glucose scaffolding (Hirschmann *et al.*, (1992) *J. Amer. Chem. Soc.* 114: 92179218), analogous organic syntheses of small compound libraries (Chen *et al.* (1994) *J. Amer. Chem. Soc.* 116: 2661), oligocarbamates (Cho, *et al.*, (1993) *Science* 261:1303), and/or peptidyl phosphonates (Campbell *et al.*, (1994) *J. Org. Chem.* 59: 658). *See, generally,* Gordon *et al.*, (1994) *J. Med. Chem.* 37:1385, nucleic acid libraries, peptide nucleic acid libraries (*see, e.g.*, U.S. Patent 5,539,083) antibody libraries (*see, e.g.*, Vaughn *et al.* (1996) *Nature Biotechnology*, 14(3): 309-314), and PCT/US96/10287), carbohydrate libraries (*see, e.g.*, Liang *et al.* (1996) *Science*, 274: 1520-1522, and U.S. Patent 5,593,853), and small organic molecule libraries (*see, e.g.*, benzodiazepines, Baum (1993) C&EN, Jan 18, page 33, isoprenoids U.S. Patent 5,569,588, thiazolidinones and metathiazanones U.S. Patent 5,549,974, pyrrolidines U.S. Patents 5,525,735 and

5,519,134, morpholino compounds U.S. Patent 5,506,337, benzodiazepines 5,288,514, and the like).

Devices for the preparation of combinatorial libraries are commercially available (*see, e.g.*, 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn,

- 5 MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA).

A number of well known robotic systems have also been developed for solution phase chemistries. These systems include automated workstations like the automated synthesis apparatus developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and many

- 10 robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, Mass.; Orca, HewlettPackard, Palo Alto, Calif.) which mimic the manual synthetic operations performed by a chemist. Any of the above devices are suitable for use with the present invention. The nature and implementation of modifications to these devices (if any) so that they can operate as discussed herein will be apparent to persons skilled in the relevant art.
- 15 In addition, numerous combinatorial libraries are themselves commercially available (*see, e.g.*, ComGenex, Princeton, N.J., Asinex, Moscow, Ru, Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, *etc.*).

High throughput assays of chemical libraries

- 20 Any of the assays for compounds capable of modulating potassium ion channel proteins described herein are amenable to high throughput screening. High throughput screening systems are commercially available (*see, e.g.*, Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH; Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA, *etc.*). These systems typically automate entire procedures
- 25 including all sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate in detector(s) appropriate for the assay. These configurable systems provide high throughput and rapid start up as well as a high degree of flexibility and customization. The manufacturers of such systems provide detailed protocols the various high throughput. Thus, for example, Zymark Corp. provides technical bulletins describing
- 30 screening systems for detecting the modulation of gene transcription, ligand binding, and

the like.

Assays for modulation of potassium flow.

- The activity of functional potassium channels of this invention can be assessed using a variety of *in vitro* and *in vivo* assays, e.g., measuring voltage, current, measuring membrane potential, measuring ion flux, e.g., potassium or rubidium, measuring potassium concentration, measuring second messengers and transcription levels, and using e.g., voltage-sensitive dyes, radioactive tracers, and patch-clamp electrophysiology. In particular such assays can be used to test for modulators both inhibitors and activators of channels.
- 5 10 Modulators of the potassium channels are tested using biologically active, functional two-transmembrane domain type potassium ion channels, either recombinant or naturally occurring. In recombinantly based assays, the subunits are typically expressed and modulation is tested using one of the *in vitro* or *in vivo* assays described below.

- In brief, samples or assays that are treated with a potential channel inhibitors or activators 15 are compared to control samples without the test compound, to examine the extent of modulation. Control samples e.g., those untreated with activators or inhibitors are assigned a relative potassium channel activity value of 100. Inhibition is present when potassium channel activity value relative to the control is about 90%, preferably 50%, more preferably 25%. Activation of channels is achieved when the select potassium channel 20 activity value relative to the control is 110%, more preferably 150%, more preferable 200% higher.

Changes in ion flux may be assessed by determining changes in polarization (i.e., electrical potential) of the cell or membrane expressing the potassium channels of this invention. A preferred means to determine changes in cellular polarization is by measuring changes in current (thereby measuring changes in polarization) with voltage-clamp and patch-clamp techniques, e.g., the "cell-attached" mode, the "inside-out" mode, and the "whole cell" mode (see, e.g., Ackerman *et al.*, *New Engl. J. Med.* 336:1575-1595 (1997)). Whole cell currents are conveniently determined using the standard methodology (see, e.g., Hamil *et al.*, *Pflugers Archiv.* 391:85 (1981). Other known assays include: radiolabeled rubidium

flux assays and fluorescence assays using voltage-sensitive dyes (see, e.g., Vestergaard-Bogind *et al.*, *J. Membrane Biol.* 88:67-75 (1988); Daniel *et al.*, *J. Pharmacol. Meth.* 25:185-193 (1991); Holevinsky *et al.*, *J. Membrane Biology* 137:59-70 (1994)). Assays for compounds capable of inhibiting or increasing potassium flux through 5 the channel proteins can be performed by application of the compounds to a bath solution in contact with and comprising cells having an channel of the present invention (see, e.g., Blatz *et al.*, *Nature* 323:718-720 (1986); Park, *J. Physiol.* 481:555-570 (1994)). Generally, the compounds to be tested are present in the range from 1 pM to 100 mM.

- 10 The effects of the test compounds upon the function of the channels can be measured by changes in the electrical currents or ionic flux or by the consequences of changes in currents and flux. Changes in electrical current or ionic flux are measured by either increases or decreases in flux of cations such as potassium or rubidium ions. The cations can be measured in a variety of standard ways. They can be measured directly by 15 concentration changes of the ions or indirectly by membrane potential or by radiolabeling of the ions. Consequences of the test compound on ion flux can be quite varied. Accordingly, any suitable physiological change can be used to assess the influence of a test compound on the channels of this invention. The effects of a test compound can be measured by a toxin binding assay. When the functional consequences are determined 20 using intact cells or animals, one can also measure a variety of effects such as transmitter release (e.g., dopamine), hormone release (e.g., insulin), transcriptional changes to both known and uncharacterized genetic markers (e.g., northern blots), cell volume changes (e.g., in red blood cells), immunoresponses (e.g., T cell activation), changes in cell metabolism such as cell growth or pH changes, and changes in intracellular second 25 messengers such as $[Ca^{2+}]$.

Prokaryotic cation channel protein mutated to mimic a functional eukaryotic cation channel protein

- Furthermore, as explained above, the present invention extends to prokaryotic cation channel proteins mutated to mimic a functional eukaryotic cation channel protein. These 30 mutated cation channel proteins have broad applications in assays for screening potential

drugs or therapeutic agents which potentially can interact with eukaryotic cation channel proteins, and be used to treat numerous conditions related to the function of cation channel proteins *in vivo*, such as cardiac arrhythmia, diabetes mellitus, seizure disorder, asthma or hypertension, to name only a few.

- 5 Presently available recombinant DNA techniques, such as site directed mutagenesis for example, can be used to readily mutate one or a number of codons of an isolated nucleic acid molecule encoding A prokaryotic cation channel protein which can then be expressed to produce a mutated prokaryotic cation channel protein which mimics a eukaryotic cation channel protein.

10

- Furthermore, prokaryotic cation channel proteins having applications in this aspect of the present invention comprise prokaryotic potassium channel proteins, prokaryotic sodium channel proteins, or prokaryotic calcium channel proteins. Such prokaryotic cation channel proteins can be obtained from varying prokaryotic organisms, such as *E. coli*, *Streptomyces* 15 *lividans*, *Clostridium acetobutylicum*, or *Staphylococcus aureus*, to name only a few. More specifically, a prokaryotic potassium channel protein comprising an amino acid sequence of SEQ ID NOs:1, 2, 3, or 7, or conserved variants thereof, can be mutated to mimic the physiological functions and chemical properties of numerous eukaryotic cation channel proteins. In a preferred embodiment, a potassium channel protein from *Streptomyces* 20 *lividans* is mutated to mimic the physiological functions and chemical properties of a eukaryotic cation channel protein, such as a eukaryotic potassium channel protein, a eukaryotic sodium channel protein, or a eukaryotic calcium channel protein. Consequently, a potential drug or agent which interacts with a mutated prokaryotic channel protein of the present invention, such as binding thereto for example, should undergo the same or similar 25 interactions with a eukaryotic cation channel protein the prokaryotic cation channel protein was mutated to mimic. Hence, a mutated prokaryotic cation channel protein of the present invention can serve as a model for a specific eukaryotic cation channel protein in screening potential drugs or therapeutic agents for interaction therewith.

- Moreover, pursuant to the present invention, and using recombinant DNA techniques, a 30 prokaryotic cation channel protein can be mutated to mimic eukaryotic cation channel

proteins from numerous eukaryotic organisms, such as, for example, insects or mammals. More specifically, a prokaryotic cation channel protein can be mutated to mimic eukaryotic cation channel proteins from a wide variety of eukaryotic organisms, such as *Drosophila melanogaster*, *Homo sapiens*, *C. elegans*, *Mus musculus*, *Arabidopsis thaliana*, or *Rattus novegicus*, to name only a few. Such eukaryotic cation channel proteins comprise an amino acid sequence comprising SEQ ID Nos: 4, 5, 6, 8, 9, 10, 11, 12, 13, or 14, or conserved variants thereof.

In a preferred embodiment of the present invention, the prokaryotic cation channel protein comprises a potassium channel protein from *Streptomyces lividans* comprising an amino acid sequence of SEQ ID NO:1, or conserved variants thereof, which is mutated to comprise an amino acid sequence of SEQ ID NO:16, or conserved variants thereof, in order to mimic the physiological functions and chemical properties of a eukaryotic cation channel protein comprising an amino acid sequence of SEQ ID NO:4. Moreover, such a mutated prokaryotic cation channel protein of the present invention is encoded by an isolated nucleic acid molecule comprising a DNA sequence of SEQ ID NO:17, or degenerate variants thereof.

Mutant cation Channel Protein

Moreover, the present invention is directed to a mutant cation channel protein. More specifically, the present invention comprises a mutant potassium channel protein comprising an amino acid sequence of SEQ ID NO:16, or conserved variants thereof.

The nomenclature used to define the polypeptides is that specified by Schroder & Lubke, "The Peptides", Academic Press (1965), wherein in accordance with conventional representation the amino group at the N-terminal appears to the left and the carboxyl group at the C-terminal to the right. NH₂ refers to the amide group present at the carboxy terminus when written at the right of a polypeptide sequence.

Accordingly, conserved variants of an isolated mutant cation channel protein of the present invention displaying substantially equivalent activity to an isolated cation channel protein of the present invention, are likewise contemplated for use in the present invention. These modifications can be obtained through peptide synthesis utilizing the appropriate starting

material.

In keeping with standard polypeptide nomenclature, *J. Biol. Chem.*, 243:3552-59 (1969), abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

5	<u>SYMBOL</u>	<u>AMINO ACID</u>	
		<u>1-Letter</u>	<u>3-Letter</u>
	Y		Tyr tyrosine
	G		Gly glycine
	F		Phe phenylalanine
10	M		Met methionine
	A		Ala alanine
	S		Ser serine
	I		Ile isoleucine
	L		Leu leucine
15	T		Thr threonine
	V		Val valine
	P		Pro proline
	K		Lys lysine
	H		His histidine
20	Q		Gln glutamine
	E		Glu glutamic acid
	W		Trp tryptophan
	R		Arg arginine
	D		Asp aspartic acid

N	Asn	asparagine
C	Cys	cysteine

It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues. The above Table is presented to correlate the three-letter and one-letter notations which may appear alternately herein.

Hence, an amino acid in the mutant cation channel protein of the present invention can be changed in a non-conservative manner (i.e., by changing an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (i.e., by changing an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less change in the structure and function of the resulting polypeptide. The present invention should be considered to include analogs whose sequences contain conservative changes which do not significantly alter the activity or binding characteristics of the resulting polypeptide.

The following is one example of various groupings of amino acids:

20 Amino acids with nonpolar R groups

Alanine

Valine

Leucine

Isoleucine

25 Proline

Phenylalanine

Tryptophan

Methionine

Amino acids with uncharged polar R groups

Glycine

Serine

5 Threonine

Cysteine

Tyrosine

Asparagine

Glutamine

10 Amino acids with charged polar R groups (negatively charged at pH 6.0)

Aspartic acid

Glutamic acid

Basic amino acids (positively charged at pH 6.0)

Lysine

15 Arginine

Histidine (at pH 6.0)

Another grouping may be those amino acids with aromatic groups:

Phenylalanine

Tryptophan

20 Tyrosine

Another grouping may be according to molecular weight (i.e., size of R groups):

Glycine 75

Alanine 89

Serine	105
Proline	115
Valine	117
Threonine	119
5 Cysteine	121
Leucine	131
Isoleucine	131
Asparagine	132
Aspartic acid	133
10 Glutamine	146
Lysine	146
Glutamic acid	147
Methionine	149
Histidine (at pH 6.0)	155
15 Phenylalanine	165
Arginine	174
Tyrosine	181
Tryptophan	204

Particularly preferred substitutions are:

20 - Gln for Arg or Lys; and

- His for Lys or Arg.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys, or with a carrier of the present invention. A His may 25 be introduced as a particularly "catalytic" site (i.e., His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of

its particularly planar structure, which induces β -turns in the polypeptide's structure.

Alternately, D-amino acids can be substituted for the L-amino acids at one or more positions.

Antibodies to an isolated mutant cation channel protein of the invention

- 5 As explained above, the present invention further extends to antibodies of a cation channel protein of the present invention, or conserved variants thereof. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. The anti-mutant channel cation protein antibodies of the invention may be cross reactive, e.g., they may recognize cation channel proteins from different species, and even different types of cation channel proteins, i.e. potassium, sodium, calcium channel proteins, or their numerous variants which are gated with different mechanisms (i.e. voltage-gated, mechanical gated, ligand binding gated, etc.). Polyclonal antibodies have greater likelihood of cross reactivity.
- 10
- 15
- 20
- 25

Various procedures known in the art may be used for the production of polyclonal

- 15 antibodies to an isolated mutant cation channel protein, or conserved variants thereof, of the present invention. For the production of antibody, various host animals can be immunized by injection with a mutant cation channel protein, or conserved variants thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc. Furthermore, a mutant cation channel protein, or conserved variants thereof, of the present invention, may be conjugated to an immunogenic carrier, e.g., bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*.
- 20
- 25

- For preparation of monoclonal antibodies directed toward a mutant cation channel protein of the present invention, or conserved variants thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler and
- 30

- Milstein [*Nature* 256:495-497 (1975)], as well as the trioma technique, the human B-cell hybridoma technique [Kozbor et al., *Immunology Today* 4:72 1983); Cote et al., *Proc. Natl. Acad. Sci. U.S.A.* 80:2026-2030 (1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole et al., in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)]. In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology [PCT/US90/02545]. In fact, according to the invention, techniques developed for the production of "chimeric antibodies" [Morrison et al., *J. Bacteriol.* 159:870 (1984); Neuberger et al., *Nature* 312:604-608 (1984); Takeda et al., *Nature* 314:452-454 (1985)]
5 by splicing the genes from a mouse antibody molecule specific for an isolated mutant cation channel protein of the present invention, or conserved variants thereof, together with a fragment of a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.
10

- According to the invention, techniques described for the production of single chain antibodies [U.S. Patent Nos. 5,476,786 and 5,132,405 to Huston; U.S. Patent 4,946,778] can be adapted to produce single chain antibodies specific for an isolated mutant cation channel protein of the invention or conserved variants thereof. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries [Huse et al., *Science* 246:1275-1281 (1989)] to allow rapid and easy identification
15 of monoclonal Fab fragments with the desired specificity for an isolated mutant cation channel protein of the present invention, or conserved variants thereof.
20

- Antibody fragments which contain the idiotype of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragment which can be produced by pepsin digestion of the antibody molecule; the
25 Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

- In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, e.g., radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using
30

- colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by
- 5 detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. For example, to select antibodies which recognize a specific epitope of an
- 10 isolated mutant cation channel protein of the present invention, or conserved variants thereof, one may assay generated hybridomas for a product which binds to a fragment of an isolated mutant cation channel protein, or conserved variants thereof, containing such epitope. For selection of an
- 15 The foregoing antibodies can be used in methods known in the art relating to the localization and activity of an isolated mutant cation channel protein, or conserved variants thereof, *e.g.*, for Western blotting, imaging such a cation channel protein *in situ*, measuring levels thereof in appropriate physiological samples, etc. using any of the detection techniques mentioned above or known in the art.
- 20 In a specific embodiment, antibodies that agonize or antagonize the activity of an isolated mutant cation channel protein of the present invention, or conserved variants thereof, can be generated. Such antibodies can be tested using the assays described *infra* for identifying ligands.

25 Detectably labeled antibodies of an isolated mutant cation channel protein of the present invention, or conserved variants thereof

Moreover, the present invention extends to antibodies described above, detectably labeled. Suitable detectable labels include enzymes, radioactive isotopes, fluorophores (*e.g.*, fluorescene isothiocyanate (FITC), phycoerythrin (PE), Texas red (TR), rhodamine, free or chelated lanthanide series salts, especially Eu³⁺, to name a few fluorophores).

chromophores, radioisotopes, chelating agents, dyes, colloidal gold, latex particles, ligands (e.g., biotin), and chemiluminescent agents. When a control marker is employed, the same or different labels may be used for the receptor and control marker.

In the instance where a radioactive label, such as the isotopes ^3H , ^{14}C , ^{32}P , ^{35}S , ^{36}Cl , ^{51}Cr ,
5 ^{57}Co , ^{58}Co , ^{59}Fe , ^{90}Y , ^{125}I , ^{131}I , and ^{186}Re are used, known currently available counting
procedures may be utilized. In the instance where the label is an enzyme, detection may be
accomplished by any of the presently utilized colorimetric, spectrophotometric,
fluorospectrophotometric, amperometric or gasometric techniques known in the art.

Direct labels are one example of labels which can be used according to the present
10 invention. A direct label has been defined as an entity, which in its natural state, is readily
visible, either to the naked eye, or with the aid of an optical filter and/or applied
stimulation, e.g. U.V. light to promote fluorescence. Among examples of colored labels,
which can be used according to the present invention, include metallic sol particles, for
example, gold sol particles such as those described by Leuvering (U.S. Patent 4,313,734);
15 dye sole particles such as described by Gribnau et al. (U.S. Patent 4,373,932) and May et
al. (WO 88/08534); dyed latex such as described by May, *supra*, Snyder (EP-A 0 280 559
and 0 281 327); or dyes encapsulated in liposomes as described by Campbell et al. (U.S.
Patent 4,703,017). Other direct labels include a radionucleotide, a fluorescent moiety or a
luminescent moiety. In addition to these direct labeling devices, indirect labels comprising
20 enzymes can also be used according to the present invention. Various types of enzyme
linked immunoassays are well known in the art, for example, alkaline phosphatase and
horseradish peroxidase, lysozyme, glucose-6-phosphate dehydrogenase, lactate
dehydrogenase, urease, these and others have been discussed in detail by Eva Engvall in
Enzyme Immunoassay ELISA and EMIT in *Methods in Enzymology*, 70. 419-439, 1980
25 and in U.S. Patent 4,857,453.

Suitable enzymes include, but are not limited to, alkaline phosphatase and horseradish peroxidase.

Other labels for use in the invention include magnetic beads or magnetic resonance imaging labels.

- As explained above, the present invention contemplates an isolated nucleic molecule, or degenerate variants thereof, which encode a mutant cation channel protein, or conserved variants thereof. Accordingly, with the present invention, there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within
- 5 the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); *DNA Cloning: A Practical Approach*, Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid*
- 10 *Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).
- 15 Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an

20 autonomous unit of DNA replication *in vivo*, i.e., capable of replication under its own control.

A "cassette" refers to a segment of DNA that can be inserted into a vector at specific restriction sites. The segment of DNA encodes a polypeptide of interest, and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading

25 frame for transcription and translation.

A cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. A cell has been "transformed" by exogenous or heterologous DNA when the transfected DNA effects a phenotypic change. Preferably, the transforming DNA should be integrated (covalently linked) into chromosomal DNA making

up the genome of the cell.

"Heterologous" DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell.

- A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides
- 5 (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular
- 10 DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according
- 15 to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.
- 20 A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (*see* Sambrook et al., *supra*). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For
- 25 preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T_m of 55°, can be used, *e.g.*, 5x SCC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SCC, 0.5% SDS). Moderate stringency hybridization conditions correspond to a higher T_m , *e.g.*, 40% formamide, with 5x or 6x SCC. High stringency hybridization conditions correspond to the highest T_m , *e.g.*, 50%
- 30 formamide, 5x or 6x SCC. Hybridization requires that the two nucleic acids contain

- complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between
- 5 two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (*see* Sambrook et al., *supra*, 9.50-0.51). For hybridization with shorter
- 10 nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (*see* Sambrook et al., *supra*, 11.7-11.8). Preferably a minimum length for a hybridizable nucleic acid is at least about 12 nucleotides; preferably at least about 18 nucleotides; and more preferably the length is at least about 27 nucleotides; and most preferably 36 nucleotides.
- 15 In a specific embodiment, the term "standard hybridization conditions" refers to a T_m of 55°C, and utilizes conditions as set forth above. In a preferred embodiment, the T_m is 60°C; in a more preferred embodiment, the T_m is 65°C.

- A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of
- 20 appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended
- 25 for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

- 30 A "promoter sequence" or "promoter" is a DNA regulatory region capable of binding

- RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

- A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced and translated into the protein encoded by the coding sequence.

- As used herein, the term "sequence homology" in all its grammatical forms refers to the relationship between proteins that possess a "common evolutionary origin," including proteins from superfamilies (*e.g.*, the immunoglobulin superfamily) and homologous proteins from different species (*e.g.*, myosin light chain, etc.) [Reeck *et al.*, *Cell*, **50**:667 (1987)].

- Accordingly, the term "sequence similarity" in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that do not share a common evolutionary origin [*see Reeck et al., 1987, supra*]. However, in common usage and in the instant application, the term "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and not a common evolutionary origin.

- In a specific embodiment, two DNA sequences are "substantially homologous" or "substantially similar" when at least about 50% (preferably at least about 75%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of

the art. See, e.g., Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

- Similarly, in a particular embodiment, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 30% of the amino acids are identical, or greater than about 60% are similar (functionally identical). Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, Version 7, Madison, Wisconsin) pileup program.

- The term "corresponding to" is used herein to refer similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. Thus, the term "corresponding to" refers to the sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

- Moreover, due to degenerate nature of codons in the genetic code, a mutant cation channel protein of the present invention can be encoded by numerous isolated nucleic acid molecules. "Degenerate nature" refers to the use of different three-letter codons to specify a particular amino acid pursuant to the genetic code. It is well known in the art that the following codons can be used interchangeably to code for each specific amino acid:

20	Phenylalanine (Phe or F)	UUU or UUC
	Leucine (Leu or L)	UUA or UUG or CUU or CUC or CUA or CUG
	Isoleucine (Ile or I)	AUU or AUC or AUA
	Methionine (Met or M)	AUG
	Valine (Val or V)	GUU or GUC or GUA or GUG
25	Serine (Ser or S)	UCU or UCC or UCA or UCG or AGU or AGC
	Proline (Pro or P)	CCU or CCC or CCA or CCG

	Threonine (Thr or T)	ACU or ACC or ACA or ACG
	Alanine (Ala or A)	GCU or GCG or GCA or GCG
	Tyrosine (Tyr or Y)	UAU or UAC
	Histidine (His or H)	CAU or CAC
5	Glutamine (Gln or Q)	CAA or CAG
	Asparagine (Asn or N)	AAU or AAC
	Lysine (Lys or K)	AAA or AAG
	Aspartic Acid (Asp or D)	GAU or GAC
	Glutamic Acid (Glu or E)	GAA or GAG
10	Cysteine (Cys or C)	UGU or UGC
	Arginine (Arg or R)	CGU or CGC or CGA or CGG or AGA or AGG
	Glycine (Gly or G)	GGU or GGC or GGA or GGG
	Tryptophan (Trp or W)	UGG
	Termination codon	UAA (ochre) or UAG (amber) or UGA (opal)
15	It should be understood that the codons specified above are for RNA sequences. The corresponding codons for DNA have a T substituted for U.	

Furthermore, the present invention extends to an isolated nucleic acid molecule, or degenerate variants thereof encoding a mutant cation channel protein, detectably labeled, and a detectably labeled isolated nucleic acid molecule hybridizable under standard hybridization conditions to an isolated nucleic acid molecule, or degenerate variants thereof, encoding a cation channel protein of the present invention. Suitable detectable labels include enzymes, radioactive isotopes, fluorophores (*e.g.*, fluorescein isothiocyanate (FITC), phycoerythrin (PE), Texas red (TR), rhodamine, free or chelated lanthanide series salts, especially Eu³⁺, to name a few fluorophores), chromophores, radioisotopes, chelating agents, dyes, colloidal gold, latex particles, ligands (*e.g.*, biotin), and chemiluminescent agents. When a control marker is employed, the same or different labels may be used for the receptor and control marker.

In the instance where a radioactive label, such as the isotopes ^3H , ^{14}C , ^{32}P , ^{35}S , ^{36}Cl , ^{51}Cr , ^{57}Co , ^{58}Co , ^{59}Fe , ^{90}Y , ^{125}I , ^{131}I , and ^{186}Re are used, known currently available counting procedures may be utilized. In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, 5 fluorospectrophotometric, amperometric or gasometric techniques known in the art.

- Direct labels are one example of labels which can be used according to the present invention. A direct label has been defined as an entity, which in its natural state, is readily visible, either to the naked eye, or with the aid of an optical filter and/or applied stimulation, e.g. U.V. light to promote fluorescence. Among examples of colored labels, 10 which can be used according to the present invention, include metallic sol particles, for example, gold sol particles such as those described by Leuvering (U.S. Patent 4,313,734); dye sole particles such as described by Gribnau et al. (U.S. Patent 4,373,932) and May et al. (WO 88/08534); dyed latex such as described by May, *supra*, Snyder (EP-A 0 280 559 and 0 281 327); or dyes encapsulated in liposomes as described by Campbell et al. (U.S. 15 Patent 4,703,017). Other direct labels include a radionucleotide, a fluorescent moiety or a luminescent moiety. In addition to these direct labeling devices, indirect labels comprising enzymes can also be used according to the present invention. Various types of enzyme linked immunoassays are well known in the art, for example, alkaline phosphatase and horseradish peroxidase, lysozyme, glucose-6-phosphate dehydrogenase, lactate 20 dehydrogenase, urease, these and others have been discussed in detail by Eva Engvall in Enzyme Immunoassay ELISA and EMIT in *Methods in Enzymology*, 70. 419-439, 1980. and in U.S. Patent 4,857,453.

Suitable enzymes include, but are not limited to, alkaline phosphatase and horseradish peroxidase.

- 25 Other labels for use in the invention include magnetic beads or magnetic resonance imaging labels.

Cloning Vectors

The present invention also extends to cloning vectors comprising an isolated nucleic acid molecule of the present invention, or degenerate variants thereof, and an origin of

replication. For purposes of this Application, an "origin of replication refers to those DNA sequences that participate in DNA synthesis.

As explained above, in an embodiment of the present invention, an isolated nucleic acid molecule, or degenerate variants thereof, encoding a mutant cation channel protein of the 5 present invention, along with isolated nucleic acid molecules hybridizable under standard hybridization conditions to an isolated nucleic acid, or degenerate variants thereof, which encodes a mutant cation channel protein of the present invention, can be inserted into an appropriate cloning vector in order to produce multiple copies of the isolated nucleic acid. A large number of vector-host systems known in the art may be used. Possible vectors 10 include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Examples of vectors include, but are not limited to, *E. coli*, bacteriophages such as lambda derivatives, or plasmids such as pBR322 derivatives or pUC plasmid derivatives, e.g., pGEX vectors, pmal-c, pFLAG, etc. The insertion into a cloning vector can, for example, be accomplished by ligating an isolated nucleic acid 15 molecule of the present invention or degenerate variants thereof, or an isolated nucleic acid hybridizable thereto under standard hybridization conditions, into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the isolated nucleic acid or degenerate variants thereof, or an isolated nucleic acid hybridizable thereto under standard hybridization conditions, are not present in the 20 cloning vector, the ends of the isolated nucleic acid molecule or degenerate variants thereof, or an isolated nucleic acid molecule hybridizable under standard hybridization conditions thereto may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction 25 endonuclease recognition sequences. Such recombinant molecules can then be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of an isolated nucleic acid molecule of the present invention, or degenerate variants thereof, or an isolated nucleic acid molecule hybridizable thereto under standard hybridization conditions, can be generated. Preferably, the cloned isolated nucleic 30 acid molecule is contained on a shuttle vector plasmid, which provides for expansion in a cloning cell, e.g., *E. coli*, and facile purification for subsequent insertion into an appropriate expression cell line, if such is desired. For example, a shuttle vector, which is a vector that can replicate in more than one type of organism, can be prepared for

replication in both *E. coli* and *Saccharomyces cerevisiae* by linking sequences from an *E. coli* plasmid with sequences from the yeast 2 μ plasmid.

In an alternative method, an isolated nucleic acid molecule of the present invention, or degenerate variants thereof, or an isolated nucleic acid molecule hybridizable thereto under standard hybridization conditions may be identified and isolated after insertion into a suitable cloning vector in a "shot gun" approach. Enrichment for an isolated nucleic acid molecule, for example, by size fractionation, can be done before insertion into the cloning vector.

Expression Vectors

10 As stated above, the present invention extends to an isolated nucleic acid molecule encoding a mutant cation channel protein of the present invention, degenerate variants thereof, or an isolated nucleic acid hybridizable thereto under standard hybridization conditions.

Isolated nucleic acid molecules of the present invention can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. Such elements are termed herein a "promoter." Thus, an isolated nucleic acid molecule, or degenerate variants thereof, which encodes a mutant cation channel protein of the present, along with isolated nucleic acid molecules hybridizable thereto under standard hybridization conditions is operatively associated with a promoter in an expression vector of the invention. A DNA sequence is "operatively associated" to an expression control sequence, such as a promoter, when the expression control sequence controls and regulates the transcription and translation of that DNA sequence. The term "operatively associated" includes having an appropriate start signal (e.g., ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If an isolated nucleic acid molecule of the present invention does not contain an appropriate start signal, such a start signal can be inserted into the expression vector in front of (5' of) the isolated nucleic acid molecule.

Both cDNA and genomic sequences can be cloned and expressed under control of such regulatory sequences. An expression vector also preferably includes a replication origin.

- The necessary transcriptional and translational signals can be provided on a recombinant expression vector, or they may be supplied by the native gene encoding the wild type 5 variant of a mutant cation channel protein of the present invention, and/or its flanking regions.

- Potential host-vector systems include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors; or 10 bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

- Moreover, an isolated nucleic acid molecule of the present invention may be expressed 15 chromosomally, after integration of the coding sequence by recombination. In this regard, any of a number of amplification systems may be used to achieve high levels of stable gene expression (*See* Sambrook et al., 1989, *supra*).

- A unicellular host containing a recombinant vector comprising an isolated nucleic acid 20 molecule, or degenerate variants thereof, which encodes a mutant cation channel protein of the present invention, or an isolated nucleic acid molecule hybridizable under standard hybridization conditions to an isolated nucleic acid molecule, or degenerate variants thereof, which encodes a mutant cation channel protein of the present invention, is cultured in an appropriate cell culture medium under conditions that provide for expression of the isolated nucleic acid molecule by the cell.

- 25 Any of the methods previously described for the insertion of DNA fragments into a cloning vector may be used to construct expression vectors comprising an isolated nucleic acid molecule of the present invention, and appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombination (genetic recombination).

Expression of an isolated nucleic acid molecule of the present invention, degenerate variants thereof, or an isolated nucleic acid molecule hybridizable thereto under standard hybridization conditions, along with a an isolated mutant cation channel protein encoded by isolated nucleic acid molecules of the present invention, degenerate variants thereof, or an 5 isolated nucleic acid molecule hybridizable thereto under standard hybridization conditions, may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be used to control expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, *Nature* 290:304-310), the promoter contained in the 3' long 10 terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, *Cell* 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, *Proc. Natl. Acad. Sci. U.S.A.* 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, *Nature* 296:39-42); prokaryotic expression vectors such as the β -lactamase promoter (Villa-Kamaroff, et al., 1978, *Proc. Natl. Acad. Sci. U.S.A.* 75:3727-3731), or the *tac* promoter 15 (DeBoer, et al., 1983, *Proc. Natl. Acad. Sci. U.S.A.* 80:21-25); see also "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and the animal transcriptional control regions, which exhibit tissue specificity and have been 20 utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, *Cell* 38:639-646; Ornitz et al., 1986, *Cold Spring Harbor Symp. Quant. Biol.* 50:399-409; MacDonald, 1987, *Hepatology* 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, *Nature* 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et 25 al., 1984, *Cell* 38:647-658; Adames et al., 1985, *Nature* 318:533-538; Alexander et al., 1987, *Mol. Cell. Biol.* 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, *Cell* 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, *Genes and Devel.* 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, *Mol. Cell. Biol.* 5:1639-1648; Hammer et al., 1987, *Science* 235:53-58), alpha 1- 30 antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, *Genes and Devel.* 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, *Nature* 315:338-340; Kollias et al., 1986, *Cell* 46:89-94), myelin basic protein gene control region which is active in oligodendrocyte cells in the brain

(Readhead et al., 1987, Cell 48:703-712), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

- 5 Expression vectors comprising an isolated nucleic acid molecule, or degenerate variants thereof, encoding a mutant cation channel protein of the present invention, or an expression vector comprising an isolated nucleic acid molecule hybridizable under standard hybridization conditions to an isolated nucleic acid molecule of the present invention, can be identified by four general approaches: (a) PCR amplification of the desired plasmid
- 10 DNA or specific mRNA, (b) nucleic acid hybridization, (c) presence or absence of selection marker gene functions, and (d) expression of inserted sequences. In the first approach, the nucleic acids can be amplified by PCR to provide for detection of the amplified product. In the second approach, the presence of a foreign gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are
- 15 homologous to an inserted marker gene. In the third approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "selection marker" gene functions (e.g., β -galactosidase activity, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. In another
- 20 example, if an isolated nucleic acid of the present invention, or degenerate variants thereof, which encode a mutant cation channel protein of the present invention or conserved variants thereof, or an isolated nucleic acid molecule hybridizable thereto under standard hybridization conditions, is inserted within the "selection marker" gene sequence of the vector, recombinants containing the insert can be identified by the absence of the inserted
- 25 gene function. In the fourth approach, recombinant expression vectors can be identified by assaying for the activity, biochemical, or immunological characteristics of the gene product expressed by the recombinant, provided that the expressed protein assumes a functionally active conformation.

Production of a mutant cation channel protein of the present invention

- 30 Moreover, the present invention extends to a method of producing a mutant cation channel protein comprising an amino acid sequence of SEQ ID NO:16, or conserved variants

thereof. More specifically, a method of the present invention comprises the steps of culturing a unicellular host either transformed or transfected with an expression vector of the present invention explained above, under conditions that provide for expression of the mutant cation channel protein, and recovering the mutant cation channel protein from the
5 transformed or transfected unicellular host. As explained above, the conditions which provide for expression of a mutant channel protein of the present invention are dependent upon the expression vector and promoter used to transform or transfect a unicellular host of the invention. Since the conditions needed relative to the promoter used are within the knowledge of one of ordinary skill in this art, conditions for specific promoters are not
10 repeated here.

Moreover, collection of a cation channel protein of the present invention produced pursuant to the method stated above, is also within the knowledge of a skilled artisan.

Crystal of a cation Channel protein

As explained above, the present invention extends to a crystal of a cation channel protein
15 having a central pore, which is found natively in a lipid bilayer membrane of an animal cell, such that the central pore communicates with extracellular matrix and cellular cytosol, wherein the crystal effectively diffracts x-rays to a resolution of greater than 3.2 angstroms.

Moreover, the present invention extends to a crystal of a cation channel protein as described above, wherein the cation channel protein comprises a first layer of aromatic
20 amino acid residues positioned to extend into the lipid bilayer membrane proximate to the interface an extracellular matrix and lipid bilayer membrane, a second layer of aromatic amino acid residues positioned to extend into the lipid bilayer membrane proximate to the interface of cellular cytosol and said lipid bilayer membrane, a tetramer of four identical transmembrane subunits, and a central pore formed by the four identical transmembrane
25 subunits.

Furthermore, each transmembrane subunit comprises an inner transmembrane alpha-helix which has a kink therein, an outer transmembrane alpha-helix, and a pore alpha-helix, wherein each subunit is inserted into the tetramer of the cation channel protein so that the outer transmembrane helix of each subunit contacts the first and second layers of aromatic

- amino acid residues described above, and abuts the lipid bilayer membrane. Moreover, the inner transmembrane helix of each subunit abuts the central pore of the cation channel protein, contacts the first and second layers of aromatic amino acid residues, is tilted by about 25° with respect to the normal of the lipid bilayer membrane, and is packed against
- 5 inner transmembrane alpha helices of other transmembrane subunits at the second layer of aromatic amino acid residues forming a bundle at the second layer. The pore alpha-helix of each subunit is located at the first layer of said aromatic amino acid residues, and positioned between inner transmembrane alpha-helices of adjacent subunits, and are directed, in an amino to carboxyl sense, towards a point near the center of the central pore.
- 10 It has been further determined, based on examination of a crystal of the present invention, that the central pore of a cation channel protein, comprises a pore region located at the first layer of aromatic amino acid residues, and connected to the inner and outer transmembrane alpha-helices of said subunits. More particularly, the pore region comprises about 25-45 amino acid residues, a turret connected to the pore alpha-helix and the outer alpha-helix,
- 15 wherein the turret is located at the interface of said extracellular matrix and the lipid bilayer membrane. The pore region further comprises an ion selectivity filter connected to the pore alpha-helix and the inner transmembrane alpha-helix of each subunit. The ion selectivity filter extends into the central pore of the cation channel protein, and comprises a signature amino acid residue sequence having main chain atoms which create a stack of
- 20 sequential oxygen atoms along the selectivity filter that extend into the central pore, and amino acid residues having side chains that interact with the pore helix. It is the signature sequence which enables a cation channel protein to discriminate among the cation intended to permeate the protein, and other cations, so that only the cation intended to permeate the channel protein is permitted to permeate.

25

The central pore further comprises a tunnel into the lipid bilayer membrane which communicates with the cellular cytosol, and a cavity located within the lipid bilayer membrane between the pore region and the tunnel, and connected to the them, such that the central pore crosses the membrane.

- 30 Furthermore, the structure of all ion channel proteins share common features, which are set

forth in the crystal of a cation channel protein described above. Consequently, the present invention extends to a crystal of a cation channel protein having a central pore, which is described above, wherein the cation is selected from the group consisting of: Na⁺, K⁺, and Ca²⁺. Hence, the present invention extends to crystals of potassium channel proteins,
5 sodium channel proteins, and calcium ion channels, to name only a few. In a preferred embodiment, the crystal of a cation channel protein comprises a crystal of a potassium ion channel protein.

In addition, a crystal of an ion channel protein of a present invention can comprise an amino acid sequence of any presently known, or subsequently discovered cation protein
10 channel. Consequently, the present invention extends to a crystal of a cation channel protein having a central pore, which is found natively in a lipid bilayer membrane of an animal cell, such that the central pore communicates with extracellular matrix and cellular cytosol, wherein the crystal comprises an amino acid sequence of:

- | | | |
|----|------------------------------------|--|
| | residues 23 to 119 of SEQ ID NO:1 | (<i>Streptomyces lividans</i>); |
| 15 | residues 61 to 119 of SEQ ID NO:2 | (<i>E. coli</i>); |
| | residues 61 to 119 of SEQ ID NO:3 | (<i>Clostridium acetobutylicum</i>); |
| | residues 61 to 119 of SEQ ID NO:4 | (<i>Drosophila melanogaster</i>); |
| | residues 61 to 119 of SEQ ID NO:5 | (<i>Homo sapiens</i>); |
| | residues 61 to 119 of SEQ ID NO:6 | (<i>Homo sapiens</i>); |
| 20 | residues 61 to 119 of SEQ ID NO:7 | (<i>Paramecium tetraurelia</i>); |
| | residues 61 to 119 of SEQ ID NO:8 | (<i>C. elegans</i>); |
| | residues 61 to 119 of SEQ ID NO:9 | (<i>Mus musculus</i>); |
| | residues 61 to 119 of SEQ ID NO:10 | (<i>Homo sapiens</i>); |
| | residues 61 to 119 of SEQ ID NO:11 | (<i>Arabidopsis thaliana</i>); |
| 25 | residues 61 to 119 of SEQ ID NO:12 | (<i>Homo sapiens</i>); |
| | residues 61 to 119 of SEQ ID NO:13 | (<i>Rattus norvegicus</i>); or |
| | residues 61 to 119 of SEQ ID NO:14 | (<i>Homo sapiens</i>); |

or conserved variants thereof.

In a preferred embodiment, a crystal of the present invention having a central pore, which is found natively in a lipid bilayer membrane of an animal cell, such that the central pore communicates with extracellular matrix and cellular cytosol, comprises an amino sequence 5 of amino acid residues 23 to 119 of SEQ ID NO:1, has a space grouping of C2, and a unit cell of dimensions of $a=128.8 \text{ \AA}$, $b=68.9\text{\AA}$, $c=112.0 \text{ \AA}$, and $\beta=124.6^\circ$. Moreover, preferably, the present invention extends to a crystal as described above, wherein the cation K^+ .

Furthermore, the present invention extends to a crystal of a cation channel protein having 10 a central pore, which is found natively in a lipid bilayer membrane of an animal cell, such that the central pore communicates with extracellular matrix and cellular cytosol, wherein the channel protein comprises a signature sequence comprising:

Thr-Val-Gly-Tyr-Gly-Asp (SEQ ID NO:15).

Method for growing a crystal of the present invention

15 The present invention further extends to a method for growing a crystal of a cation channel protein having a central pore, which is found natively in a lipid bilayer membrane of an animal cell, such that the central pore communicates with extracellular matrix and cellular cytosol, by sitting-drop vapor diffusion. Such a method of the present invention comprises the steps of providing the cation channel protein, removing a predetermined number of 20 carboxy terminal amino acid residues from the cation channel protein to form a truncated cation channel protein, dissolving the truncated cation channel protein in a protein solution, such that the concentration of dissolved truncated channel protein is about 5 to about 10 mg/ml, and mixing equal volumes of protein solution with reservoir mixture at 20 ° C. Preferably, the reservoir mixture comprises 200 mM $CaCl_2$, 100 mM Hepes, 48 % PEG 25 400, pH 7.5, and the protein solution comprises (150 mM KCl, 50 mM Tris, 2 mM DTT, pH 7.5).

Moreover, the present invention extends to a method of growing a crystal of a cation channel protein as described above, wherein a crystal can be grown comprising any type of

cation channel protein. In particular, the present invention can be used to grow crystals of potassium channel proteins, sodium channel proteins, or calcium channel proteins, to name only a few.

Furthermore, the present invention extends to a method of growing a crystal of a cation channel protein, as described herein, wherein the crystal comprises an amino acid sequence of:

- residues 23 to 119 of SEQ ID NO:1 (*Streptomyces lividans*);
 - residues 61 to 119 of SEQ ID NO:2 (*E. coli*);
 - residues 61 to 119 of SEQ ID NO:3 (*Clostridium acetobutylicum*);
 - 10 residues 61 to 119 of SEQ ID NO:4 (*Drosophila melanogaster*);
 - residues 61 to 119 of SEQ ID NO:5 (*Homo sapiens*);
 - residues 61 to 119 of SEQ ID NO:6 (*Homo sapiens*);
 - residues 61 to 119 of SEQ ID NO:7 (*Paramecium tetraurelia*);
 - residues 61 to 119 of SEQ ID NO:8 (*C. elegans*);
 - 15 residues 61 to 119 of SEQ ID NO:9 (*Mus musculus*);
 - residues 61 to 119 of SEQ ID NO:10 (*Homo sapiens*);
 - residues 61 to 119 of SEQ ID NO:11 (*Arabidopsis thaliana*);
 - residues 61 to 119 of SEQ ID NO:12 (*Homo sapiens*);
 - residues 61 to 119 of SEQ ID NO:13 (*Rattus norvegicus*); or
 - 20 residues 61 to 119 of SEQ ID NO:14 (*Homo sapiens*);
- or conserved variants thereof.

Use of Crystal of a cation channel protein in assay systems for screening drugs and agents

In another embodiment, the present invention extends to a method of using a crystal of a

cation channel protein, as described herein, in an assay system for screening drugs and other agents for their ability to modulate the function of a cation channel protein, comprising the steps of initially selecting a potential drug or agent by performing rational drug design with the three-dimensional structure determined for a crystal of the present invention, wherein the selecting is performed in conjunction with computer modeling.

After potential drugs or agents have been selected, a cation channel protein is contacted with the potential drug or agent. If the drug or therapeutic agent has potential use for modulating the function of a cation channel protein, a change in the function of the cation channel after contact with the agent, relative to the function of a similar cation channel protein not contacted with the agent, or the function of the same cation channel protein prior to contact with the agent. Hence, the change in function is indicative of the ability of the drug or agent to modulate the function of a cation channel protein.

Furthermore, the present invention extends to a method of using a crystal of a cation channel protein as described herein, in an assay system for screening drugs and other agents for their ability to modulate the function of a cation channel protein, wherein the crystal comprises a Na⁺ channel protein, a K⁺ channel protein, or a Ca²⁺ channel protein.

The present invention further extends to a method of using a crystal of a cation channel protein in an assay for screening drugs or other agents for their ability to modulate the function of a cation channel protein, wherein the crystal of the cation channel protein comprises an amino acid sequence of:

residues 23 to 119 of SEQ ID NO:1	(<i>Streptomyces lividans</i>);
residues 61 to 119 of SEQ ID NO:2	(<i>E. coli</i>);
residues 61 to 119 of SEQ ID NO:3	(<i>Clostridium acetobutylicum</i>);
residues 61 to 119 of SEQ ID NO:4	(<i>Drosophila melanogaster</i>);
25 residues 61 to 119 of SEQ ID NO:5	(<i>Homo sapiens</i>);
residues 61 to 119 of SEQ ID NO:6	(<i>Homo sapiens</i>);
residues 61 to 119 of SEQ ID NO:7	(<i>Paramecium tetraurelia</i>);
residues 61 to 119 of SEQ ID NO:8	(<i>C. elegans</i>);

- residues 61 to 119 of SEQ ID NO:9 (*Mus musculus*);
residues 61 to 119 of SEQ ID NO:10 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:11 (*Arabidopsis thaliana*);
residues 61 to 119 of SEQ ID NO:12 (*Homo sapiens*);
5 residues 61 to 119 of SEQ ID NO:13 (*Rattus norvegicus*); or
residues 61 to 119 of SEQ ID NO:14 (*Homo sapiens*);
or conserved variants thereof.

In a preferred embodiment of a method of using a crystal of a cation channel protein in an assay for screening drugs or other agents for their ability to modulate the function of a 10 cation channel protein, the crystal comprises a potassium channel protein, comprising amino acid residues 23 to 119 of SEQ ID NO:1, a space grouping of C2, and a unit cell of dimensions of $a=128.8 \text{ \AA}$, $b=68.9\text{\AA}$, $c=112.0 \text{ \AA}$, and $\beta=124.6^\circ$.

Moreover, it is important to note that a drug's or agent's ability to modulate the function of a cation channel protein includes, but is not limited to, increasing or decreasing the cation 15 channel protein's permeability to the specific cation relative the permeability of the same or a similar not contacted with the drug or agent, or the same cation channel protein prior to contact with the drug or agent.

In a further embodiment, the present invention extends to a method of using a crystal of a cation channel protein, as set forth herein, in an assay system for screening drugs and other 20 agents for their ability to treat conditions related to the function of cation channel proteins *in vivo*, and particularly in abnormal cellular control processes related to the functioning of cation channel protein. Such a method comprises the initial step of selecting a potential drug or other agent by performing rational drug design with the three-dimensional structure determined for a crystal of the invention, wherein the selecting is performed in conjunction 25 with computer modeling. After potential drugs or therapeutic agents are selected, a cation channel protein is contacted with the potential drug or agent. If an interaction of the potential drug or other agent with the cation channel is detected, it is indicative of the potential use of the drug or agent to treat conditions related the function of cation channel proteins *in vivo*. Examples of such conditions include, but are not limited to, cardiac

arrhythmia, diabetes mellitus, seizure disorder, asthma or hypertension, to name only a few.

Furthermore, a crystal of a cation channel protein used in the method for screening drugs or agents for their ability to interact with a cation channel comprises an Na⁺ channel 5 protein, K⁺ channel protein, or Ca²⁺ channel protein. Hence, the method of the present invention can be used to screen drugs or agents capable of treating conditions related to the function of such channels.

Moreover, the present invention extends to a crystal used in the method for screening drugs or agents for their ability to interact with a cation channel protein comprising an amino acid 10 sequence of:

residues 23 to 119 of SEQ ID NO:1 (*Streptomyces lividans*);
residues 61 to 119 of SEQ ID NO:2 (*E. coli*);
residues 61 to 119 of SEQ ID NO:3 (*Clostridium acetobutylicum*);
residues 61 to 119 of SEQ ID NO:4 (*Drosophila melanogaster*);
15 residues 61 to 119 of SEQ ID NO:5 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:6 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:7 (*Paramecium tetraurelia*);
residues 61 to 119 of SEQ ID NO:8 (*C. elegans*);
residues 61 to 119 of SEQ ID NO:9 (*Mus musculus*);
20 residues 61 to 119 of SEQ ID NO:10 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:11 (*Arabidopsis thaliana*);
residues 61 to 119 of SEQ ID NO:12 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:13 (*Rattus norvegicus*); or
residues 61 to 119 of SEQ ID NO:14 (*Homo sapiens*),
25 or conserved variants thereof.

In a preferred embodiment, a crystal used in a method for screening drugs or agents for

their ability to interact with a cation channel, comprises amino acid residues 23 to 119 of SEQ ID NO:1, has a space grouping of C2, and a unit cell of dimensions of $a=128.8 \text{ \AA}$, $b=68.9\text{\AA}$, $c=112.0 \text{ \AA}$, and $\beta=124.6^\circ$.

- In yet another embodiment, the present invention extends to a method of using a crystal of
- 5 a cation channel protein described herein, in an assay system for screening drugs and other agents for their ability to permeate through a cation channel protein, comprising an initial step of selecting a potential drug or other agent by performing rational drug design with the three-dimensional structure determined for the crystal, wherein the selecting of the potential drug or agent is performed in conjunction with computer modeling. After a potential drug
- 10 or agent has been selected, a cation channel protein can be prepared for use in the assay. For example, preparing the cation channel protein can include isolating the cation channel protein from the membrane of a cell, and then inserting the cation channel protein into a membrane having a first and second side which is impermeable to the potential drug or agent. As a result, the cation channel protein traverses the membrane, such that the
- 15 extracellular portion of the cation channel protein is located on the first side of the membrane, and the intracellular portion of the cation channel protein is located on the second side of the membrane. The extracellular portion of the cation channel membrane can then be contacted with the potential drug or agent. The presence of the drug or agent in the second side of the membrane is indicative of the drug's or agent's potential to permeate
- 20 the cation channel protein, and the drug or agent is selected based on its ability to permeate the cation channel protein.

In addition, a crystal used in a method for screening drugs or agents for their ability to permeate a cation channel can comprise a Na^+ channel protein, a K^+ protein channel, or a Ca^{2+} protein channel.

- 25 Furthermore, the present invention extends to the use of a crystal in an assay system for screening drugs and other agents for their ability to permeate through a cation channel protein, wherein the crystal comprises an amino acid sequence of:

residues 23 to 119 of SEQ ID NO:1 (*Streptomyces lividans*);

residues 61 to 119 of SEQ ID NO:2 (*E. coli*);

residues 61 to 119 of SEQ ID NO:3 (*Clostridium acetobutylicum*);
residues 61 to 119 of SEQ ID NO:4 (*Drosophila melanogaster*);
residues 61 to 119 of SEQ ID NO:5 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:6 (*Homo sapiens*);
5 residues 61 to 119 of SEQ ID NO:7 (*Paramecium tetraurelia*);
residues 61 to 119 of SEQ ID NO:8 (*C. elegans*);
residues 61 to 119 of SEQ ID NO:9 (*Mus musculus*);
residues 61 to 119 of SEQ ID NO:10 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:11 (*Arabidopsis thaliana*);
10 residues 61 to 119 of SEQ ID NO:12 (*Homo sapiens*);
residues 61 to 119 of SEQ ID NO:13 (*Rattus novegicus*); or
residues 61 to 119 of SEQ ID NO:14 (*Homo sapiens*);

or conserved variants thereof.

In a preferred embodiment, the crystal used in an assay system of the present invention for screening drugs and other agents for their ability to permeate through a cation channel protein comprises amino acid residues 23 to 119 of SEQ ID NO:1, has a space grouping of C2, and a unit cell of dimensions of $a=128.8 \text{ \AA}$, $b=68.9\text{\AA}$, $c=112.0 \text{ \AA}$, and $\beta=124.6^\circ$.

In the assay systems disclosed herein, Once the three-dimensional structure of a crystal comprising a cation channel protein is determined, a potentia drugs and therapeutic agents 20 which may interact with a carrier channel protein, i.e. bind or modulate the function thereof, or perhaps be able to permeate through such a protein can be examined through the use of computer modeling using a docking program such as GRAM, DOCK, or AUTODOCK [Dunbrack *et al.*, 1997, *supra*]. This procedure can include computer fitting of potential drugs or agents to a cation channel protein to ascertain how well the shape and 25 the chemical structure of the potential drug or agent will complement or interact with a cation channel protein. [Bugg *et al.*, *Scientific American*, Dec.:92-98 (1993); West *et al.*, *TIPS*, 16:67-74 (1995)]. Computer programs can also be employed to estimate the attraction, repulsion, and steric hindrance of a potential drug or agent to a cation channel

protein. Generally the tighter the fit, the lower the steric hindrances, and the greater the attractive forces, the more potent the potential drug or agent, since these properties are consistent with a tighter binding, and are clearly indicative of an interaction with a cation channel protein. Furthermore, the more specificity in the design of a potential drug the 5 more likely that the drug will not interact as well with other proteins. This will minimize potential side-effects due to unwanted interactions with other proteins.

- Furthermore, computer modeling programs based on the structure of a cation channel protein in a crystal of the present invention, can be used to modify potential drugs or agents in order to identify potentially more promising drugs. Such analysis has been 10 shown to be effective in the development of HIV protease inhibitors [Lam *et al.*, *Science* 263:380-384 (1994); Wlodawer *et al.*, *Ann. Rev. Biochem.* 62:543-585 (1993); Appelt, *Perspectives in Drug Discovery and Design* 1:23-48 (1993); Erickson, *Perspectives in Drug Discovery and Design* 1:109-128 (1993)]. Alternatively a potential drug or agent can be obtained by initially screening a random peptide library produced by recombinant 15 bacteriophage for example, [Scott and Smith, *Science*, 249:386-390 (1990); Cwirla *et al.*, *Proc. Natl. Acad. Sci.*, 87:6378-6382 (1990); Devlin *et al.*, *Science*, 249:404-406 (1990)]. A peptide selected in this manner would then be systematically modified by computer modeling programs in order to enhance its potential interaction with a cation channel protein.
- 20 Moreover, through the use of the three-dimensional structure disclosed herein and computer modeling, a large number of these compounds can be rapidly screened on the computer monitor screen, and a few likely candidates can be determined without the laborious synthesis of untold numbers of compounds.
- Once a potential drug or agent is identified, it can be either selected from a library of 25 chemicals as are commercially available from most large chemical companies including Merck, GlaxoWelcome, Bristol Meyers Squib, Monsanto/Searle, Eli Lilly, Novartis and Pharmacia UpJohn, alternatively the potential drug or agent may be synthesized *de novo*. The *de novo* synthesis of one or even a relatively small group of specific compounds is reasonable in the art of drug design. The potential drug or agent can then be placed into an 30 assay of the present invention to determine whether it binds with a cation channel protein.

When suitable potential drugs or agents are identified, a supplemental crystal is grown which comprises a cation channel protein. Preferably the crystal effectively diffracts X-rays for the determination of the atomic coordinates of the protein-ligand complex to a resolution of greater than 5.0 Angstroms, more preferably greater than 3.0 Angstroms, and even more preferably greater than 2.0 Angstroms. The three-dimensional structure of the supplemental crystal is determined by Molecular Replacement Analysis. Molecular replacement involves using a known three-dimensional structure as a search model to determine the structure of a closely related molecule or protein-ligand complex in a new crystal form. The measured X-ray diffraction properties of the new crystal are compared with the search model structure to compute the position and orientation of the protein in the new crystal. Computer programs that can be used include: X-PLOR and AMORE [J. Navaza, *Acta Crystallographics ASO*, 157-163 (1994)]. Once the position and orientation are known an electron density map can be calculated using the search model to provide X-ray phases. Thereafter, the electron density is inspected for structural differences and the search model is modified to conform to the new structure.

The present invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention.

They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE I

Potassium Channel Structure: Molecular Basis of K⁺ Conduction and Selectivity

The K⁺ channel from *Streptomyces lividans* is an integral membrane protein with sequence similarity to all known K⁺ channels, particularly in the pore region. X-ray analysis with data to 3.2 Å reveals that four identical subunits create an inverted tepee, or cone, cradling the selectivity filter of the pore in its outer end. The narrow selectivity filter is only 12 Å long, while the remainder of the pore is wider and lined with hydrophobic amino acids. A large, water-filled cavity and helix dipoles are positioned so as to overcome electrostatic destabilization of an ion in the pore at the center of the bilayer. Main-chain carbonyl oxygen atoms from the K⁺ channel signature sequence line the selectivity filter, which is

held open by structural constraints to coordinate K⁺ ions but not smaller Na⁺ ions. The selectivity filter contains two K⁺ ions about 7.5 Å apart. This configuration promotes ion conduction by exploiting electrostatic repulsive forces to overcome attractive forces between K⁺ ions and the selectivity filter. The architecture of the pore establishes the 5 physical principles underlying selective K⁺ conduction.

- More particularly, potassium ions diffuse rapidly across cell membranes through proteins called K⁺ channels, which underlie many fundamental biological processes including electrical signaling in the nervous system. Potassium channels use diverse mechanisms of gating (the processes by which the pore opens and closes), but they all exhibit very similar 10 ion permeability characteristics (1). All K⁺ channels show a selectivity sequence of K⁺ ≈ Rb⁺ > Cs⁺, while permeability for the smallest alkali metal ions Na⁺ and Li⁺ is immeasurably low. Potassium is at least ten thousand times more permeant than Na⁺, a feature that is essential to the function of K⁺ channels. Potassium channels also share a constellation of permeability characteristics that is indicative of a multi-ion conduction 15 mechanism: The flux of ions in one direction shows high order coupling to flux in the opposite direction, and ionic mixtures result in anomalous conduction behavior (2). Because of these properties, K⁺ channels are classified as "long pore channels", invoking the notion that multiple ions queue inside a long, narrow pore in single-file fashion. In addition, the pores of all K⁺ channels can be blocked by tetraethylammonium ions (3).
- 20 Molecular cloning and mutagenesis experiments have reinforced the conclusion that all K⁺ channels have essentially the same pore constitution. Without exception, they contain a critical amino acid sequence that has been termed the K⁺ channel signature sequence. Mutation of these amino acids disrupts the channel's ability to discriminate between K⁺ and Na⁺ ions (4).
- 25 Biophysicists have been tantalized for the past quarter century about chemical basis of the impressive fidelity with which the channel distinguishes between K⁺ and Na⁺ ions, which are featureless spheres of Pauling radius 1.33 Å and 0.95 Å and the ability of K⁺ channels to be concurrently so highly selective and exhibit a throughput rate approaching the diffusion limit. The 10⁴ margin by which K⁺ is selected over Na⁺ implies strong energetic 30 interactions between K⁺ ions and the pore. And yet strong energetic interactions seem incongruent with throughput rates up to 10⁸ ions per second.

Potassium Channel Architecture

Amino acid sequences show the relationship of the K⁺ channel from *Streptomyces lividans* (kcsa K⁺ channel) (5) to other channels in biology, including vertebrate and invertebrate voltage-dependent K⁺ channels, vertebrate inward rectifier and Ca²⁺-activated K⁺ channels,

5 K⁺ channels from plants and bacteria, and cyclic nucleotide-gated cation channels (Fig. 1). On the basis of hydrophobicity analysis, there are two closely related varieties of K⁺ channels, those containing two membrane-spanning segments per subunit and those containing six. In all cases, the functional K⁺ channel protein is a tetramer (6), typically of four identical subunits (7). Subunits of the two membrane-spanning variety appear to be

10 shortened versions of their larger counterparts, as if they simply lack the first four membrane-spanning segments. Though the kcsa K⁺ channel belongs to the two membrane-spanning set of K⁺ channels, its amino acid sequence is actually closer to those of eukaryotic six membrane-spanning K⁺ channels. In particular, its sequence in the pore region, located between the membrane-spanning stretches and containing the K⁺ channel

15 signature sequence, is nearly identical to that found in the *Drosophila (Shaker)* and vertebrate voltage-gated K⁺ channels (Fig. 1). Moreover, through a study of the kcsa K⁺ channel interaction with eukaryotic K⁺ channel toxins, as described *infra*, it has been confirmed that the kcsa K⁺ pore structure is indeed very similar to that of eukaryotic K⁺ channels, and that its structure is maintained when it is removed from the membrane using

20 detergent (8).

Furthermore, the kcsa K⁺ channel structure from residue position 23 to 119 of SEQ ID NO:1 has been determined with X-ray crystallography (Table 1). The cytoplasmic carboxyl terminus (residues 126 to 158 of SEQ ID NO:1) were removed in the preparation and the remaining residues were disordered. The kcsa K⁺ channel crystals are radiation sensitive and the diffraction pattern is anisotropic, with reflections observed along the best and worst directions at 2.5 Å and 3.5 Å Bragg spacings, respectively. By careful data selection, anisotropy correction, introduction of heavy atom sites by site-directed mutagenesis, averaging and solvent flattening, an interpretable electron density map has been calculated (Fig. 2, A-C). This map was without main chain breaks and showed strong side chain density (Fig. 2C). The model was refined with data to 3.2 Å (the data set was 93 % complete to 3.2 Å with 67% completeness between 3.3 Å and 3.2 Å), maintaining highly restrained stereochemistry and keeping tight noncrystallographic symmetry

restraints. The refinement procedure was monitored by minimizing the value R-free (29.0%) and its separation from R-crystallographic (28.0%). The presence of four molecules (subunits) in the asymmetric unit of the crystal provides a very significant enhancement of the accuracy of the crystallographic analysis; firstly, by enabling averaging 5 of the electron density over four crystallographically independent regions of the MIR map, and secondly, by providing a powerful set of constraints on the atomic model during refinement (9).

The K⁺ channel is a tetramer with four-fold symmetry about a central pore (Fig. 3, A and B). Like several other membrane proteins it has two layers of aromatic amino acids 10 positioned to extend into the lipid bilayer, presumably near the membrane-water interfaces (Fig. 3C) (10). Each subunit has two transmembrane α -helices connected by the roughly 30 amino acid pore-region consisting of the turret, pore helix, and selectivity filter (Fig. 3 A and B). A subunit is inserted into the tetramer such that one transmembrane helix (inner helix) faces the central pore while the other (outer helix) faces the lipid membrane. The 15 inner helices are tilted with respect to the membrane normal by about 25° and are slightly kinked as shown, so that the subunits open like the petals of a flower facing the outside of the cell. The open petals house the structure formed by the pore region near the extracellular surface of the membrane. This region contains the K⁺ channel signature sequence, which forms the selectivity filter (4). The essential features of subunit packing 20 can be appreciated by viewing the relation between the four inner helices and the four pore helices (Fig. 3D). The four inner helices pack against each other as a bundle near the intracellular aspect of the membrane, giving the appearance of an inverted tepee. The pore helices are slotted in between the poles of the tepee and are directed, with an amino to carboxyl sense, towards a point near the center of the channel (Fig. 3, A,B, and D). This 25 pore helix arrangement is significant in that it provides many of the intersubunit contacts that hold the tetramer together and, as discussed below, is also critical in the operation of the ion conduction pore.

Sequence conservation among K⁺ channels (including ones with two and six membrane-spanning segments), as well as cyclic nucleotide-gated cation channels, is 30 strongest for the amino acids corresponding to the pore region and the inner helix. Even Na⁺ and Ca²⁺ channels show distant relatedness over these segments (not shown). It has been concluded that the tepee architecture of the K⁺ channel pore is a general feature of all

of cation channel proteins, and that they all will have four inner helices arranged like the poles of a tepee, four pore helices, and a selectivity filter - tuned to select the appropriate cation - located close to the extracellular surface.

Surprisingly, this structure of the kcsa K⁺ channel is in excellent agreement with extensive functional and mutagenesis studies on *Shaker* and other eukaryotic K⁺ channels (Fig. 4). The pore-region of K⁺ channels was first discovered with pore-blocking scorpion toxins (11). These inhibitors interact with amino acids (white) comprising the broad extracellular-facing entryway to the pore (12). The impermeant organic cation tetraethylammonium (TEA) blocks K⁺ channels from both sides of the membrane at distinct sites (13). Amino acids interacting with externally and internally applied TEA are located just external to (yellow) and internal to (mustard) the structure formed by the signature sequence amino acids (14, 15). Alteration of the signature sequence amino acids (red main chain atoms) disrupts K⁺ selectivity (4). Amino acids close to the intracellular opening on the *Shaker* K⁺ channel map to the inner helix on the kcsa K⁺ channel (16). Interestingly, exposure to the cytoplasm of the region above the inner helix bundle (pink side chains) requires an open voltage-dependent gate, whereas the region at or below the bundle (green side chains) is exposed whether or not the gate was open. The correlation between the transition zone for gate dependent exposure to the cytoplasm in the *Shaker* K⁺ channel and the inner helix bundle in this structure has implications for mechanisms of gating in K⁺ channels.

General Properties of the Ion Conduction Pore

Both the intracellular and extracellular entryways are charged negative by acidic amino acids (Fig. 5A, red), an effect that would raise the local concentration of cations while lowering the concentration of anions. The overall length of the pore is about 45 Å and its diameter varies along its distance (Fig. 5B). From inside the cell (bottom) the pore begins as a tunnel about 18 Å in length (the internal pore) and then opens into a wide cavity (about 10 Å across) near the middle of the membrane. A K⁺ ion could move throughout the internal pore and cavity and still remain mostly hydrated. In contrast, the selectivity filter separating the cavity from the extracellular solution is so narrow that a K⁺ ion would have to shed its hydrating waters to enter. The chemical composition of the wall lining the internal pore and cavity is predominantly hydrophobic (Fig. 5A, yellow). The selectivity

filter, on the other hand, is lined exclusively by polar main chain atoms belonging to the signature sequence amino acids. The distinct mechanisms operating in the cavity and internal pore versus the selectivity filter are discussed below.

As explained above, potassium channel proteins exclude the smaller alkali metal cations

- 5 Li⁺ (radius 0.60 Å) and Na⁺ (0.95 Å) but allow permeation of the larger members of the series Rb⁺ (1.48 Å) and Cs⁺ (1.69 Å). In fact Rb⁺ is nearly the perfect K⁺ (1.33 Å) analog as its size and permeability characteristics are very similar to those of K⁺. Because they are more electron dense than K⁺, Rb⁺ and Cs⁺ allow visualization of the locations of permeant ions in the pore. By difference electron density maps calculated with data from
- 10 crystals transferred into Rb⁺-containing (Fig. 6A) or Cs⁺-containing (Fig. 6B) solutions, multiple ions are well-defined in the pore. The selectivity filter contains two ions (inner and outer ions) located at opposite ends, about 7.5 Å apart (center to center). In the Rb⁺ difference map, there actually are two partially separated peaks at the inner aspect of the selectivity filter. These peaks are too close to each other (2.6 Å) to represent two
- 15 simultaneously occupied ion binding sites. Although Applicant is under no obligation to explain such peaks, and is not to be bound by any explanations, Applicant merely postulates these peaks may represent a single ion (on average) in rapid equilibrium between adjacent sites. The single inner ion peak in the Cs⁺ difference map undoubtedly reflects the lower resolution at which the map was calculated (to 5 Å for Cs⁺ versus 4.0 Å for Rb⁺) since the Rb⁺ difference map, when calculated at the same lower resolution, also shows only a single peak at the Cs⁺ position. The Rb⁺ positions correspond to strong peaks (presumably K⁺ ions) in a high contour native electron density map (not shown). Thus, the selectivity filter may contain two K⁺ ions. A third weaker peak is located below the
- 20 selectivity filter at the center of the large cavity in the Rb⁺ difference map (Fig. 6A, lower peak) and in the Cs⁺ difference map at lower contour (not shown). Electron density at the cavity center is prominent in MIR maps even prior to averaging (Fig. 6C, lower diffuse peak). The difference electron density maps show this to be related to the presence of one or more poorly localized cations situated at least 4 Å away from the closest protein groups.
- 25

The Cavity and Internal Pore

- 30 Figures 5B and 6 indicate that surprisingly, a 10 Å diameter cavity is in the center of the channel protein with an ion in it. Electrostatic calculations indicate that when an ion is

moved along a narrow pore through a membrane it must cross an energy barrier that is maximum at the membrane center (17). The electrostatic field emanating from a cation polarizes its environment, bringing the negative ends of dipoles closer to it and thereby stabilizing it. At the bilayer center, the polarizability of the surrounding medium is

5 minimal and therefore the energy of the cation is highest. Thus, simple electrostatic considerations allow an understanding of the functional significance of the cavity and its strategic location. The cavity will serve to overcome the electrostatic destabilization resulting from the low dielectric bilayer by simply surrounding an ion with polarizable water. A second feature of the K⁺ channel structure will also stabilize a cation at the

10 bilayer center. The four pore helices point directly at the center of the cavity (Fig. 3, A, B and D). The amino to carboxyl orientation of these helices will impose a negative electrostatic (cation attractive) potential via the helix dipole effect (18). The ends of the helices are rather far (about 8 Å) from the cavity center, but all four contribute to the effect. Therefore, two properties of the structure, the aqueous cavity and the oriented

15 helices, help to solve a very fundamental physical problem in biology - how to lower the electrostatic barrier facing a cation crossing a lipid bilayer. Thus, the diffuse electron density in the cavity center most likely reflects not an ion binding site, but rather a hydrated cation cloud (Fig. 7).

In summary, the inner pore and cavity lower electrostatic barriers without creating deep

20 energy wells. The structural and chemical design of this part of the pore ensure a low resistance pathway from the cytoplasm to the selectivity filter, facilitating a high throughput. Functional experiments on K⁺ channels support this conclusion. When TEA from the cytoplasm migrates to its binding site at the top of the cavity, >50% of the physical distance across the membrane (Fig. 4 and Fig. 5), it traverses only about 20% of

25 the transmembrane voltage difference (15). Thus, 80% of the transmembrane voltage is imposed across the relatively short selectivity filter. The rate limiting steps for a K⁺ ion traversing the channel are thereby limited to this short distance. In effect, the K⁺ channel has thinned the relevant transmembrane diffusion distance to a mere 12 Å.

The Selectivity Filter

30 Construction of the atomic model for the K⁺ channel selectivity filter was based on the experimental electron density map which showed a continuous ridge of electron density

attributable to the main chain, as well as strong valine and tyrosine side chain density directed away from the pore (Fig. 8A). K⁺ ion positions defined by difference Fourier analysis (Fig. 6 and Fig. 8A, yellow density) along with knowledge of alkali metal cation coordination in small molecules were also used in the construction. The side chain
5 locations preclude their direct participation in ion coordination, leaving this function to the main chain atoms. The precise orientation of individual carbonyl oxygens can not be discerned at the resolution of this X-ray analysis. Although Applicant is under no obligation to explain the orientation of individual carbonyl atoms, and are not to be bound by such explanations, Applicant merely proposes they are directed inward to account for
10 K⁺ ion coordination (Fig. 8B). A single water molecule (the only one modeled in the structure) located between the two K⁺ ions in the selectivity filter was justified by the presence of a strong electron density peak in the experimental map which was never associated with an ion peak in the difference Fourier maps (19).

The structure of the selectivity filter exhibits two essential features. First, the use of main
15 chain atoms creates a stack of sequential oxygen rings and thus affords numerous closely spaced sites of suitable dimensions for coordinating a dehydrated K⁺ ion. The K⁺ ion thus has only a very small distance to diffuse from one site to the next within the selectivity filter. The second important structural feature of the selectivity filter is the protein packing around it. The Val and Tyr side chains from the V-G-Y-G sequence point away from the
20 pore and make specific interactions with amino acids from the tilted pore helix. In collusion with the pore helix Trp residues, the four Tyr side chains form a massive sheet of aromatic amino acids, twelve in total, that is positioned like a cuff around the selectivity filter (Fig. 8C). The hydrogen bonding, for example between the Tyr hydroxyls and Trp nitrogens, and the extensive van der Waals contacts within the sheet, offer the immediate
25 impression that this structure behaves like a layer of springs stretched radially outward to hold the pore open at its proper diameter.

Applicant postulates, although under no obligation to do so, and not to be bound thereby, that when an ion enters the selectivity filter it evidently dehydrates (nearly completely). To compensate for the energetic cost of dehydration, the carbonyl oxygen atoms must take the
30 place of the water oxygen atoms. That is, they must come in very close contact with the ion and act like surrogate water (20, 21). The structure reveals that the selectivity filter is being held open as if to prevent it from accommodating a Na⁺ ion with its smaller radius.

Therefore, Applicant postulates that a K⁺ ion fits in the filter just right, so that the energetic costs and gains are well balanced. Sodium on the other hand is too small. The structure of the selectivity filter with its molecular springs holding it open prevents the carbonyl oxygen atoms from approaching close enough to compensate for the cost of 5 dehydration of a Na⁺ ion.

This analysis shows that the selectivity filter contains two K⁺ ions in the presence of about 150 mM K⁺ (Fig. 6 and Fig. 8). The ions are located at opposite ends of the selectivity filter, separated by about 7.5 Å. That is roughly the average distance between K⁺ ions in a 4 Molar KCl solution, and in the selectivity filter there are no intervening Cl⁻ anions to 10 balance the charge. Although under no obligation to explain such results, and without intending to be bound by any explanation, Applicant postulates, that the selectivity filter attracts and concentrates K⁺ ions. The structure implies that a single K⁺ ion would be held very tightly, but that the presence of two K⁺ ions results in mutual repulsion, hence their locations near opposite ends of the selectivity filter. Thus, when a second ion enters, the 15 attractive force between a K⁺ ion and the selectivity filter becomes perfectly balanced by the repulsive force between ions, and this is what allows conduction to occur. This picture accounts for both a strong interaction between K⁺ ions and the selectivity filter and a high throughput mediated by electrostatic repulsion. On the basis of functional measurements, the same concept of destabilization by multiple ion occupancy has been proposed for Ca²⁺ 20 channels (22) and for K⁺ channels (23) and perhaps is a general property of all selective ion channels.

Experimental Procedures

Cloning and Expression of the kcsa gene

The kcsa gene was subcloned into pQE60 (Qiagen) vector and expressed in *E. coli* XL-1 25 Blue cells upon induction with 1-β-D-thiogalactopyranoside. The carboxy-terminal histidine tagged protein was extracted by homogenization and solubilization in 40 mM decylmaltose (Antrace). The kcsa K⁺ channel was purified on a cobalt affinity column. Thirty-five carboxyl terminal amino acids were cleaved by chymotrypsin proteolysis. The truncated channel was purified to homogeneity by gel filtration and the detergent exchanged 30 in a final dialysis step against 5 mM N,N,-dimethyldodecylamine-N-oxide (LDAO). Crystals were grown at 20° C by using the sitting drop method by mixing equal volumes of

a solubilizing solution with reservoir mixture. Through the entire preparation, the channel protein was maintained in solutions containing 150 mM KCl. For definition of K⁺ sites, crystals were transferred into solutions where 150 mM KCl was replaced by 150 mM RbCl or 150 mM CsCl.

5 X-ray crystallography

Crystals (space group C2: $a = 128.8 \text{ \AA}$, $b = 68.9 \text{ \AA}$, $c = 112.0 \text{ \AA}$, $\beta = 124.6^\circ$) were flash-frozen by transferring directly from the crystal mother liquor to a stream of boiled-off nitrogen (24). Since crystals of the mutant L90C diffracted significantly better than wild type protein crystals, the former were used for native data collection. Data were collected
10 from multiple crystals and six sets were selected and merged to form the native data set used for structure determination. Mercury derivatives were obtained by direct addition of methyl mercury to the crystallization solution of cysteine mutant crystals. MALDI-TOF mass spectrometry confirmed 60-90% derivatization of crystals prior to data collection. All data were collected at Cornell High Energy Synchrotron Source (CHESS), station A1,
15 using the Princeton 2K CCD (25). Data were processed with DENZO and SCALEPACK (26) and the CCP4 package (27). Heavy atom positions were determined with SHELX-97 (28) and cross-difference Fourier analysis. These positions confirmed the four-fold noncrystallographic symmetry observed in the self-rotation Patterson function and allowed the determination of initial orientation matrices. An initial model (90% complete) was built
20 into a solvent flattened (64% solvent content), four-fold averaged electron density map using the program O (29). The tracing of the model was facilitated by the use of the mercury atom positions as residue markers. L86C was used solely for this purpose. After torsional refinement (with strict four-fold noncrystallographic symmetry constraints) using XPLOR 3.851 (30), this model was used in the anisotropic scaling (sharpening (31)) of the
25 native data with XPLOR. The structure factor sigma values were also rescaled appropriately and the corrected data were used for all subsequent procedures. Four-fold averaging, solvent flattening and phase extension were applied in DM (32), resulting in a marked improvement of the electron density that allowed correction of the model and the building of additional residues. Refinement consisted of rounds of positional (in the initial
30 stages phase information was also included as a restraint) and grouped B-factor refinement in XPLOR. Four-fold noncrystallographic symmetry was highly restrained with the force constant for positional restraints set as 1000 kcal/mol/Å². The diffuse ion cloud described

in the text was initially modeled as one or more K⁺ ions and several water molecules, however the results were unsatisfactory. Therefore, this and other strong unmodeled density present in solvent flattened maps (no averaging included) was Fourier back-transformed, scaled and included in the refinement procedure, as partial structure factors. The final model includes amino acids 23 to 119 of each chain. The following residues were truncated: Arg27 to C β , Ile60 to C γ , Arg64 to C β , Glu71 to C β and Arg117 to N ϵ . The stereochemistry is strongly restrained, with no outliers on the Ramachandran plot. The high B-factor values reflect the intensity decay of the data beyond 4 Å.

10

Summary

Without intending to be bound by such proposals, and with no obligation to explain these results, Applicant proposes the following principles underlying the structure and operation of K⁺ channels. (i) The pore structure defines an inverted tepee architecture with the selectivity filter held at its wide end. This architecture also describes the pore of cyclic nucleotide-gated channels and probably Na⁺ and Ca²⁺ channels as well. (ii) The narrow selectivity filter is only 12 Å long, while surprisingly, the remainder of the pore is wider and has a relatively inert hydrophobic lining. These structural and chemical properties favor a high K⁺ throughput by minimizing the distance over which K⁺ interacts strongly with the channel. (iii) A large water-filled cavity and helix dipoles help to overcome the high electrostatic energy barrier facing a cation in the low dielectric membrane center. (iv) The K⁺ selectivity filter is lined by carbonyl oxygen atoms providing multiple closely spaced sites. The filter is constrained in an optimal geometry so that a dehydrated K⁺ ion fits with proper coordination while the Na⁺ ion is too small. (v) Two K⁺ ions at close proximity in the selectivity filter repel each other. The repulsion overcomes the otherwise strong interaction between ion and protein and allows rapid conduction in the setting of high selectivity.

Table 1. Summary of data collection and refinement statistics.

Data Collection and Phasing:

	Dataset	Resolution (Å)	Redundancy	Completeness Overall/outer	Rmerge #	Phasing Power ¶	R-Cullis +
5	L90C-a	15.0-3.7	3.5	91.3/93.3 %	0.071	1.61	0.70
	L90C-b	15.0-3.7	7.0	91.5/94.1 %	0.083	1.87	0.50
	V93C	15.0-3.7	4.1	98.3/99.1 %	0.075	1.35	0.63
	A32C	15.0-4.0	2.3	84.1/83.8 %	0.076	1.45	0.66
	A29C	15.0-5.0	2.7	73.9/74.0 %	0.063	1.03	0.85
	A42C	15.0-6.5	2.0	90.7/90.3 %	0.057	0.97	0.81
10	L86C	30.0-6.0	2.3	58.7/58.9 %	0.057	--	--

				<i>I</i> / <i>σI</i>	% of measured data with <i>I</i> / <i>σI</i> >2
15	Native	30.0-3.2	6.1	93.3 %	0.086 15.8 79
	Outer Shell	3.3-3.2	2.3	66.6 %	0.286 3.9 50

Anisotropic correction:

		Average F.O.M*	Average F.O.M*
20		(30.0-3.2 Å)	(3.4-3.2 Å)
	Before Sharpening ∂	0.76	0.55
	After sharpening ∂	0.83	0.64

Refinement:

25	Resolution	10.0-3.2 Å	Root-mean-square deviation of bond angles: 1.09°
	R-cryst. &:	28.0 %	bond lengths 0.005 Å
	R-free &:	29.0 %	ncs related atoms: 0.006 Å
	No. of reflections with $ F /\sigma F >2$:	12054	related atoms: 10 Å ²
30	No. of protein atoms:	710 per subunit	B-factor for non- bonded atoms: 36 Å ²
	No. of ligand atoms:	1 water, 3 K ⁺ atoms	
	Mean B-factor for side-chain atoms:	90 Å ²	
35	Mean B-factor for side-chain atoms:	110 Å ²	

R_{merge} = $\sum \sum |I - \bar{I}| / \sum I$; ¶ Phasing power = $<|F_h|>/<|E|>$; -R-Cullis = $\sum ||F_{ph} \pm F_p - |F_{hc}||| / \sum |F_{ph} \pm F_p|$, only for centric data; & R-cryst. = $\sum |F_p - F_{p(calc)}| / \sum |F_p|$, r-free the same for R-cryst., but calculated on 10% of data selected in thin resolution shells and excluded from refinement; *F.O.M.: figure of merit; σ in both cases four-fold averaging and solvent flattening were applied; I_j is the observed intensity, \bar{I} is the average intensity, F_h is the root-mean-square heavy-atom structure factor, E is the lack of closure error, F_{ph} is the structure factor for the derivative, F_p is the structure factor for the native, F_{hc} is the calculated structure factor for the heavy-atom, $F_{p(calc)}$ is the calculated native structure value.

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XL-1 Blue cells upon induction with 1-(D-thiogalactopyranoside. The carboxyl-terminal
histidine tagged protein was extracted by homogenization and solubilization in 40mM
10 decylmaltoside (Anatrace). The kcsa channel was purified on a cobalt affinity column.
Thirty-five carboxyl terminal amino acids were cleaved by chymotrypsin proteolysis. The
truncated channel was purified to homogeneity by gel filtration and the detergent exchanged
in a final dialysis step against 5 mM N,N-dimethyldodecylamine-N-oxide (LDAO).
Crystals were grown at 20(C by using the sitting drop method by mixing equal volumes of
15 protein solution (5-10 mg/ml, 150 mM KCl, 50 mM Tris pH 7.5, 2 mM DTT) with
reservoir mixture (200mM CaCl₂, 100mM Hepes pH 7.5 and 48 % PEG 400). Through the
entire preparation the channel protein was maintained in solutions containing 150 mM KCl.
For definition of K⁺ sites, crystals were transferred into solutions where 150 mM KCl was
replaced by 150mM RbCl or 150mM CsCl.
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EXAMPLE IIStructural Conservation in Prokaryotic and EukaryoticK⁺ Channels Revealed by Scorpion Toxins

Scorpion toxins inhibit ion conduction through K⁺ channels by occluding the pore at their
5 extracellular opening. A single toxin protein binds very specifically to a single K⁺ channel
to cause inhibition. The toxins are 35 to 40 amino acids in length and have a characteristic
fold that is held rigidly by three disulfide bridges (1). They are active site inhibitors,
because when they bind to the channel they interact energetically with K⁺ ions in the pore
(2-4). The intimate interaction between these inhibitors and the pore of K⁺ channels has
10 been exploited to gain insights into the structure and function of K⁺ channels.

Studies employing site-directed mutagenesis of the *Shaker* K⁺ channel have mapped the
scorpion toxin binding site to regions corresponding to the extracellular entryway of the
kcsa K⁺ channel (4-9). Although the K⁺ channel selectivity filter amino acids are highly
conserved, the residues lining the entryway are quite variable. As if to mirror the amino
15 acid variation at the binding site, the toxins are also highly variable in their amino acid
composition. A given scorpion venom is a veritable library of toxins, apparently ensuring
that a scorpion will inhibit a large fraction of K⁺ channel types in its victim. Studies on
the specificity of toxin-channel interactions have led to the following understanding. The
extracellular entryway to the K⁺ channel is relatively conserved in its three-dimensional
20 structure but the precise amino acid composition is not conserved. The scorpion toxins
have a shape, dictated by their conserved fold, that enables them to fit snugly into the
entryway, but the affinity of a given toxin-channel pair depends on the residue match (or
mismatch) on both interaction surfaces.

A study of the interaction between the kcsa K⁺ channel (5) and the scorpion toxin agitoxin2
25 has been undertaken (10). By producing, through mutagenesis, a competent toxin binding
site, it is shown that the kcsa K⁺ channel pore structure and extracellular entryway is very
similar to that of eukaryotic voltage-gated K⁺ channels such as the *Shaker* K⁺ channel from
Drosophila and the vertebrate voltage-gated K⁺ channels, and that mutated potassium
channel proteins of prokaryotic organisms mimic the physiological functions and chemical
30 properties of eukaryotic cation binding proteins. By combining functional data collected on
the toxin-channel interaction with the structures of both proteins Applicant proposes,

without intending to be bound by such proposals, a highly-restrained model of the complex structure.

Experimental Procedures

- Three mutations (Q58A, T61S, R64D) were introduced into the kcsa K⁺ channel gene to
- 5 modify its pore region sequence using PCR mutagenesis and confirmed by DNA sequencing. The gene also contained a mutation at the second residue (P2A) to introduce an ncol restriction endonuclease site and it was lacking the last two carboxyl terminal residues (both Arg) to avoid proteolysis during the protein preparation. This gene was cloned into the pQE60 vector for expression with a carboxyl terminal thrombin and
- 10 hexahistidine fusion. Channel protein was expressed in XL-1 Blue strain of *E. coli* (Stratagene) by induction with 1-β-D-thiogalactopyranoside at a concentration of 1.0 mM. Three hours following induction bacteria were sonicated in 50 mM Tris buffer (7.5), 100 mM KCl, 10 mM Mg₂SO₄, 25 mg DNase 1, 250 mM sucrose, in addition to pepstatin, leupeptin, and PMSF. The channel was extracted in the same solution containg 40 mM
- 15 decylmaltoside (Anatrace) at room temperature. Following centrifugation the supernatant was bound to cobalt resin (Talon) at a protein to resin ratio that will saturate the resin. The resin was washed, and detergent concentration was lowered to 10.0 mM. One mL columns were prepared. The control resin (no channel) was handled in the same manner. The resin preparation was the same for mass spectrometry and binding studies.
- 20 Forty mg of *Leiurus quinquestriatus hebraeus* venom (Alomone Labs) was suspended in buffer identical to that of the channel (10.0 mM decylmaltoside) and applied to the column. After washing, channel was eluted with 1.0 M imidazole in the same buffer.
- Wild type and mutant agitoxin2 were prepared (10). Tritiated N-ethylmaleimide (NEN Life Sciences) was conjugated to agitoxin2 D20C (14). Binding was performed in a 300
- 25 μL volume containing 50 mM Tris (7.5), 100 mM KCl, 10 mM decylmaltoside, and 0.3 μL of cobalt resin saturated with the mutant kcsa K⁺ channel for 30 minutes at room temperature. Following brief centrifugation the supernatant was removed, resin was applied to a filter, rinsed briefly with ice cold buffer, and then counted in a scintillation counter. All binding measurements were made with a paired control containing a
- 30 saturating concentration (200 times K_D) of unlabeled wild type agitoxin2 to determine

nonspecific binding. The competition assay was carried out under the same conditions. Labeled Agitoxin2 at 0.06 μ M was always present and unlabeled toxin was added to compete with bound labeled toxin.

Discussion

5 Guided by knowledge of the toxin receptor on the *Shaker K⁺* channel, set forth in SEQ ID NO:4, three point mutations were introduced into the kcsa K⁺ channel (SEQ ID NO:1) that should render it sensitive to scorpion toxins (Fig 9). The amino acid sequence of the mutated kcsa K⁺ is set forth in SEQ ID NO:16. Amino acids 61 and 64 of SEQ ID NO:1 were changed to their *Shaker K⁺* channel counterpart, and 58 was changed to alanine since
10 a small side chain at this latter position favors binding (4, 7). The mutant kcsa K⁺ channel protein was expressed in *Escherichia coli*, extracted from the membrane with the detergent decylmaltoside, and bound to cobalt resin through a carboxyl terminal hexahistidine tag (11). A 1 mL column, prepared with the K⁺ channel-containing resin, was used to screen the venom of the Middle East scorpion *Leiurus quinquestriatus hebraeus*, the source of
15 numerous well-characterized ion channel toxins. Forty milligrams of venom was added to the column and after washing, the K⁺ channel protein was eluted with an imidazole solution (12). The eluate was analyzed with MALDI-TOF mass spectrometry, focusing on the low mass range appropriate for scorpion toxins (about 4000 Da). The K⁺ channel column resulted in a dramatic enhancement of specific peaks (Fig 10, A-C). Three of these
20 peaks corresponded in mass to the known K⁺ channel toxins agitoxin2, charybdotoxin, and Lq2 (Fig. 10, C and D). A fourth peak (Fig. 10C, asterisk) may represent a novel toxin, which is currently under study. However, Applicant is under no obligation to explain this peak, and is not bound by any theories set forth herein regarding this peak. The peak corresponding to chlorotoxin, a reported chloride channel inhibitor (13), did not bind and
25 provides an indication of the degree to which the K⁺ channel toxins are purified by the mutant kcsa K⁺ channel column (Fig. 10, A and C).

Further quantitative analysis was carried out with agitoxin2. Radiolabeled agitoxin2 was prepared by producing the mutation D20C in the toxin (located far from its channel binding surface) and conjugating it with tritiated N-ethylmaleimide (14). A filter assay showed that
30 labeled agitoxin2 binds to the mutant kcsa K⁺ channel with an equilibrium dissociation constant, K_D, of about 0.6 mM (Fig. 11A). In contrast, no binding to the wild type

channel could be detected (not shown). The total capacity of resin saturated with mutant channel protein, based on the specific activity of radiolabeled toxin and the known 1:1 stoichiometry (one toxin per tetrameric channel), is nearly 50 pmoles of channel per μ L of resin. This value approximates the expected capacity of the resin and therefore implies that 5 all of the channel in the preparation must have a correct conformation.

Amino acids in a well-defined region of agitoxin2 form its functional interaction surface, as determined by the effects of alanine substitution on binding to the *Shaker K⁺* channel [Fig. 11C (4, 8)]. Mutation of Lys 27 and Asn 30 had the largest destabilizing effects. It is noteworthy that Lys 27 is conserved in all members of this toxin family because its side 10 chain apparently plugs the pore of K⁺ channels (3). To confirm that agitoxin2 uses the same amino acids to interact with the mutant kcsa K⁺ channel, the effects of the K27A and N30A toxin mutations with a competition binding assay were studied (Fig. 11B). These mutations decreased the affinity for the toxin significantly (130-fold and 45-fold, respectively), as anticipated from the *Shaker K⁺* channel studies. In contrast, the D20C 15 mutation (predicted to be on the back side of the toxin), even with a bulky N-ethylmaleimide adduct, did not influence affinity (Fig. 11, A and B). These results indicate that agitoxin2 binds in the same manner to both the mutant prokaryotic kcsa K⁺ channel protein and the eukaryotic *Shaker K⁺* channel protein. The affinity for the *Shaker K⁺* channel is considerably higher ($K_D \sim 1$ nM), but only three amino acids have been 20 mutated in the prokaryotic cation channel protein to mimic the site on the *Shaker K⁺* channel (Fig. 9).

These results demonstrate that the overall structure of the agitoxin2 receptor site is very similar on both the kcsa and *Shaker K⁺* channels. This conclusion justifies the use of energetic data borrowed from *Shaker K⁺* channel studies to assist in the docking of 25 agitoxin2 onto the kcsa K⁺ channel structure. Thermodynamic mutant cycle analysis has allowed the identification of numerous energetically coupled residue pairs on the interface [pairs of residues that are related by the fact that mutating one influences the effect (on equilibrium binding) of mutating the other (8)]. The four best defined of these residue pairs are displayed in matched colors on the kcsa K⁺ channel and agitoxin2 surfaces (Fig 30 12 A). The three off-center residue pairs (blue, green, yellow) have the strongest mutant cycle coupling energies [> 3 kT (4, 8)]. The central residue pair (red) is coupled by 1.7 kT and independent information places Lys 27 (red residue on agitoxin2, Fig 11 A) over

the pore (3, 4). Mere visual inspection suggests a unique orientation for the toxin on the channel (Fig 12 B). If the toxin is placed with its functionally defined interaction surface face-down in the groove formed by the turrets (5), with Lys 27 at the center, the colors match well in three dimensions. The toxin seems to fit perfectly into the vestibule of a K⁺ channel. The four-fold symmetry of the K⁺ channel provides four statistically distinguishable but energetically identical orientations available for a toxin to bind [(Fig 12 A) (15)].

In summary, through a combination of structural and functional data, it is shown that prokaryotic channel proteins can be mutated to mimic the physiological functions and 10 chemical properties of eukaryotic channel proteins. Furthermore, disclosed herein is a view of a K⁺ channel in complex with a neurotoxin from scorpion venom. The kcsa K⁺ channel is structurally very similar to eukaryotic K⁺ channels. This unexpected structural conservation, determined through application of techniques developed here, can be exploited to advance our understanding of K⁺ channel pharmacology, and prepare mutant 15 prokaryotic channel proteins that can be used to screen potential drugs or agents that may interact with eukaryotic cation channel proteins in vivo, and treat conditions related to the function of proteins.

References

The following references, along with other relevant information was cited in Example II, 20 and set forth below. All references cited in Example II are hereby incorporated by reference in their entirety.

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11. Three mutations (Q58A, T61S, R64D) were introduced into the kcsa K⁺ channel gene to modify its pore region sequence using PCR mutagenesis and confirmed by DNA sequencing. The gene also contained a mutation at the second residue (P2A) to introduce an ncol restriction endonuclease site and it was lacking the last two carboxyl terminal residues. This gene was cloned into the pQE60 vector for expression with a carboxyl terminal thrombin and hexahistidine fusion. Channel protein was expressed in XL-1 Blue strain of *E. coli* (Stratagene) by induction with 1-β-D-thiogalactopyranoside at a concentration of 1.0 mM. Three hours following induction bacteria were sonicated in 50 mM Tris buffer (7.5), 100 mM KCl, 10 mM Mg₂SO₄, 25 mg DNase 1, 250 mM sucrose, in addition to pepstatin, leupeptin, and PMSF. The channel was extracted in the same solution containg 40 mM decylmaltoside (Anatrace) at room temperature. Following centrifugation the supernatant was bound to cobalt resin (Talon) at a protein to resin ratio that will saturate the resin. The resin was washed, and detergent concentration was lowered to 10.0 mM. One mL columns were prepared. The control resin (no channel) was handled in the same manner. The resin preparation was the same for mass spectrometry and binding studies.

12. Forty mg of *Leiurus quinquestriatus hebraeus* venom (Alomone Labs) was suspended in buffer identical to that of the channel (10.0 mM declymaltoside) and applied to the column. After washing, channel was eluted with 1.0 M imidazole in the same buffer.
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17. Wild type and mutant agitoxin2 were prepared (10). Tritiated N-ethylmaleimide
- 10 (NEN Life Sciences) was conjugated to agitoxin2 D20C (14). Binding was performed in a 300 μ L volume containing 50 mM Tris (7.5), 100 mM KCl, 10 mM declymaltoside, and 0.3 μ L of cobalt resin saturated with the mutant kcsA K^+ channel for 30 minutes at room temperature. Following brief centrifugation the supernatant was removed, resin was applied to a filter, rinsed briefly with ice cold buffer, and then counted in a scintillation
- 15 counter. All binding measurements were made with a paired control containing a saturating concentration (200 times K_d) of unlabeled wild type agitoxin2 to determine nonspecific binding. The competition assay was carried out under the same conditions. Labeled Agitoxin2 at 0.06 μ M was always present and unlabeled toxin was added to compete with bound labeled toxin.
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The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein
25 will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the

appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate,
5 and are provided for description.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1 1. A method of using a functional cation channel protein in an assay for screening
2 potential drugs or agents which interact with the cation channel protein, the method
3 comprising the steps of:

- 4 a) providing a functional cation channel protein;
- 5 b) conjugating the functional cation channel protein to a solid phase resin;
- 6 c) contacting the potential drug or agent to the functional cation channel
7 protein conjugated to the solid phase resin;
- 8 d) removing the functional cation channel protein from the solid phase resin;
9 and
- 10 e) determining whether the potential drug or agent is bound to the cation
11 channel protein.

1 2. The method of Claim 1, wherein the providing step comprises:

- 2 a) expressing an isolated nucleic acid molecule encoding the cation channel
3 protein in a unicellular host such that the cation channel protein is present
4 in the cell membrane of the unicellular host;
- 5 b) lysing the unicellular host in a solubilizing solution so that the cation
6 channel protein is solubilized in the solution; and
- 7 c) extracting the cation channel protein from the solubilizing solution with a
8 detergent.

1 3. The method of Claim 2, wherein lysing the unicellular host in a solubilizing
2 solution comprises sonicating the unicellular host in a solution comprising 50 mM Tris
3 buffer, 100 mM KCl, 10 mM MgSO₄, 25 mg DNase 1, 250 mM sucrose, pepstatin,

4 leupeptin, and PMSF, pH 7.5.

5 4. The method of Claim 2, wherein the detergent comprises 40 mM decylmaltoside.

6 5. The method of Claim 1, wherein the conjugating step comprises binding the cation
7 channel protein to a cobalt resin at protein to resin ratio that allows for saturation of the
8 resin with the cation channel protein.

9 6. The method of Claim 1, wherein the removing step comprises contacting the cation
10 channel protein conjugated to the solid phase resin to an imidazole solution.

1 7. The method of Claim 1, wherein the isolated nucleic acid molecule encoding the
2 cation channel protein comprises a DNA sequence of SEQ ID NO:17, or degenerate
3 variants thereof, or an isolated nucleic acid molecule hybridizable under standard
4 hybridization conditions to an isolated nucleic acid molecule comprising a DNA sequence
5 of SEQ ID NO:17, or degenerate variants thereof.

1 8. The method of Claim 1, wherein the potential drug or agent is a member of a
2 library of compounds, and the contacting step comprises contacting the library of
3 compounds to the functional cation channel protein conjugated to the solid phase resin.

9. The method of Claim 8, wherein the library of compounds comprises a mixture of
compounds or a combinatorial library.

1 10. The method of Claim 9, wherein the combinatorial library comprises a phage
2 display library, or a synthetic peptide library.

1 11. A prokaryotic cation channel protein mutated to mimic a functional eukaryotic
2 cation channel protein.

12. The prokaryotic cation channel protein of Claim 11, selected from the group
consisting of a potassium channel protein, a sodium channel protein, or a calcium channel
protein.

1 13. The prokaryotic cation channel protein of Claim 11, endogenously produced in a
2 prokaryotic organism selected from the group consisting of *E.coli*, *Streptomyces lividans*,
3 *Clostridium acetobutylicum*, or *Staphylococcus aureus*.

1 14. The prokaryotic cation channel protein of Claim 11, comprising an amino acid
2 sequence of SEQ ID Nos: 1, 2, 3, or 7.

1 15. The prokaryotic cation channel protein of Claim 11, wherein said prokaryotic
2 cation channel protein is a potassium channel protein from *Streptomyces lividans*.

1 16. The prokaryotic cation channel of Claim 15, encoded by a nucleic acid comprising
2 a DNA sequence of SEQ ID NO:17, or degenerate variants thereof.

1 17. The prokaryotic cation channel protein of Claim 15, comprising an amino acid
2 sequence of SEQ ID NO:1, or conserved variants thereof.

1 18. The prokaryotic cation channel protein of Claim 11, wherein the functional
2 eukaryotic cation channel protein comprises a eukaryotic potassium channel protein, a
3 eukaryotic sodium channel protein, or a eukaryotic calcium channel protein.

1 19. The prokaryotic cation channel protein of Claim 11, wherein said functional
2 eukaryotic cation channel protein is endogenously produced in a eukaryotic organism
3 comprising insects or mammals.

1 20. The prokaryotic cation channel protein of Claim 19, wherein said eukaryotic
2 organism comprises *Drosophila melanogaster*, *Homo sapiens*, *C. elegans*, *Mus musculus*,
3 *Arabidopsis thaliana*, *paramecium tetraurelia* or *Rattus norvegicus*.

1 21. The prokaryotic cation channel protein of Claim 11, mutated to mimic a eukaryotic
2 cation channel protein comprising an amino acid sequence comprising SEQ ID Nos: 4, 5,
3 6, 8, 9, 10, 11, 12, 13, or 14.

1 22. The prokaryotic cation channel protein of Claim 21, wherein said prokaryotic
2 channel protein is a potassium channel protein from *Streptomyces lividans* comprising an

3 amino acid sequence of SEQ ID NO:1, said eukaryotic cation channel is a potassium
4 channel protein comprising an amino acid sequence of SEQ ID NO:4, and said mutated
5 prokaryotic channel protein comprises an amino acid sequence of SEQ ID NO:16, or
6 conserved variants thereof.

1 23. The prokaryotic cation channel protein of Claim 22, wherein said mutated
2 porkaryotic channel protein is encoded by an isolated nucleic acid molecule comprising a
3 DNA sequence of SEQ ID NO:17, or degenerate variants thereof.

1 24. An isolated nucleic acid molecule which encodes a mutant K⁺ channel protein,
2 comprising a DNA sequence of SEQ ID NO:17, or degenerate variants thereof.

1 25. An isolated nucleic acid molecule hybridizable to the isolated nucleic acid molecule
2 of Claim 24 under standard hybridization conditions.

1 26. The isolated nucleic acid molecule of Claim 24, detectably labeled.

1 27. The isolated nucleic acid molecule of Claim 25, detectably labeled.

1 28. The detectably labeled isolated nucleic acid molecule of either of Claims 26 or 27,
2 wherein said detectable label comprises radioactive isotopes, compounds which fluoresce,
3 or enzymes.

1 29. The isolated nucleic acid molecule of either of Claims 24 or 25, which encode a
2 polypeptide comprising an amino acid sequence of SEQ ID NO:16, or conserved variants
3 thereof.

1 30. An isolated polypeptide comprising an amino acid sequence of SEQ ID NO:16, or
2 conserved variants thereof.

1 31. An antibody having a polypeptide of Claim 30 as an immunogen.

1 32. The antibody of Claim 31, wherein said antibody is a monoclonal antibody.

- 1 33. The antibody of Claim 32, wherein said antibody is a polyclonal antibody.
- 1 34. The antibody of Claim 33, wherein said antibody is a chimeric antibody.
- 1 35. The antibody of any of Claims 31-34 detectably labeled.
- 1 36. The antibody of Claim 35, wherein said detectable label comprises an enzyme, a
2 chemical which fluoresces, or a radioactive isotope.
- 1 37. A cloning vector comprising an isolated nucleic acid residue of either of Claims 24
2 or 25, and an origin of replication.
- 1 38. The cloning vector of Claim 37, wherein said cloning vector is selected from the
2 group consisting of *E. coli*, bacteriophages, plasmids, and pUC plasmid derivatives.
- 1 39. The cloning vector of Claim 37, wherein bacteriophages further comprise lambda
2 derivatives, plasmids further comprise pBR322 derivatives, and pUC plasmid derivatives
3 further comprise pGEX vectors, or pmal-c, pFLAG.
- 1 40. An expression vector comprising an isolated nucleic acid molecule of either of
2 Claims 24 or 25, operatively associated with a promoter.
- 1 41. The expression vector of Claim 40, wherein said promoter is selected from the group
2 consisting of the immediate early promoters of hCMV, early promoters of SV40, early
3 promoters of adenovirus, early promoters of vaccinia, early promoters of polyoma, late
4 promoters of SV40, late promoters of adenovirus, late promoters of vaccinia, late
5 promoters of polyoma, the *lac* the *trp* system, the *TAC* system, the *TRC* system, the major
6 operator and promoter regions of phage lambda, control regions of fd coat protein, 3-
7 phosphoglycerate kinase promoter, acid phosphatase promoter, and promoters of yeast α
8 mating factor.
- 1 42. A unicellular host transformed with an expression vector of Claim 40.
- 1 43. The unicellular host of Claim 42, wherein said host is selected from the group

2 consisting of *E. coli*, Pseudonomas, Bacillus, Streptomyces, yeast, CHO, R1.1, B-W, L-M,
3 COS1, COS7, BSC1, BSC40, BMT10 and Sf9 cells.

1 44. A method of producing a mutant cation channel protein comprising an amino acid
2 sequence of SEQ ID NO:16, or conserved variants thereof, comprising the steps of:

3 a) culturing a unicellular host of Claim 42 under conditions that provide for
4 expression of said mutant cation channel protein; and

5 b) recovering said mutant cation channel protein from said unicellular host.

1 45. A method of screening for compounds which selectively bind to a potassium ion
2 channel protein comprising:

3 (a) complexing a functional two-transmembrane-domain-type potassium ion channel
4 protein to a solid support;

5 (b) contacting the complexed protein/solid support with an aqueous solution said
6 solution containing a compound that is being screened for the ability to selectively
7 bind to the ion channel protein;

8 (c) determining whether the compound selectively binds to the ion channel protein
9 with the proviso that the potassium ion channel protein is in the form of a
10 tetrameric protein; and,

11 when the protein is mutated to correspond to the agitoxin2 docking site of a Shaker K⁺
12 channel protein by substituting amino acid residues permitting the mutated protein to bind
13 agitoxin2, the protein will bind agitoxin 2 while bound to the solid support, said
14 substituting of residues being within the 36 amino acid domain defined by -25 to +5 of the
15 selectivity filter where the 0 residue is either the phenylalanine or the tyrosine of the filter's
16 signature sequence selected from the group consisting of glycine-phenylalanine-glycine or
17 glycine-tyrosine-glycine.

1 46. A method of claim 45 wherein the solid supports are selected from the group

2 comprising: cobalt, insoluble polystyrene beads, PVDF, and polyethylene glycol.

1 47. A method of claim 45 wherein the two-transmembrane-domain-type ion channel
2 protein is a prokaryote.

1 48. A method of claim 45, wherein the two-transmembrane-domain-type ion channel
2 protein is from *Steptomyces lividans*.

1 49. A method of claim 45 wherein the two-transmembrane-domain-type ion channel
2 protein is KcsA.

1 50. A method of claim 45 wherein the two-transmembrane-domain-type ion channel
2 protein is mutated from a wild-type protein.

1 51. A method of claim 50 where the mutation is within the 36 amino acid domain
2 defined by -25 to +5 of the selectivity filter where the 0 residue is either the
3 phenylalanine or the tyrosine of the filter's signature sequence selected from the
4 group consisting of glycine-phenylalanine-glycine or glycine-tyrosine-glycine.

1 52. A method of claim 50 wherein the mutation deletes a subsequence of the native
2 amino acid sequence and replaces that the native with a subsequence from the
3 corresponding domain of a second and different ion channel protein.

1 53. A method of claim 52 wherein the second ion channel protein is from a eukaryote.

1 54. A method claim 45 wherein the aqueous solution comprises a non-ionic detergent.

1 55. A non-natural and functional two-transmembrane-domain-type potassium ion
2 channel protein wherein the non-natural protein is mutated in its amino acid
3 sequence from a corresponding natural protein whereby the mutation does not
4 prevent the non-natural protein from binding agitoxin2 when the non-natural
5 protein is further mutated to correspond to the agitoxin2 docking site of a Shaker
6 K⁺ channel protein said docking site created by substituting amino acid residues
7 selected from within the 36 amino acid domain defined by -25 to +5 of the Shaker

1 K⁺ selectivity filter where the 0 residue is either the phenylalanine or the tyrosine
2 of the filter's signature sequence selected from the group consisting of
3 glycine-phenylalanine-glycine or glycine-tyrosine-glycine.

1 56. A non-natural protein of claim 55 wherein the protein binds to a channel blocking
2 protein toxin with at least a 10 fold increase in affinity over the native ion channel.

1 57. A non-natural protein of claim 55 wherein the natural protein is the KcsA from
2 *Streptomyces lividans*.

1 58. A method of assessing the adequacy of the structural conformation of a
2 two-transmembrane-domain-type potassium ion channel protein for high through
3 put assays comprising the steps of:

4 (a) complexing a two-transmembrane-domain-type potassium ion channel protein
5 having a tetrameric form to a non-lipid solid support under aqueous conditions;

6 (b) contacting the complexed two-transmembrane-domain-type potassium ion channel
7 protein with a substance known to bind to the two-transmembrane-domain-type
8 potassium ion channel protein when bound to lipid membrane wherein the
9 substance also modulates potassium ion flow in that channel protein; and,

10 (c) detecting the binding of the substance to the complexed
11 two-transmembrane-domain-type potassium ion channel protein.

1 59. A method of claim 58 wherein the two-transmembrane-domain-type potassium ion
2 channel protein is mutated from a wild type two-transmembrane-domain-type
3 potassium ion channel protein by substitution of amino acids.

1 60. A method of claim 58 wherein the contacting is done in the presence of a non-ionic
2 detergent.

1 60. A method of claim 58 where in the substance is a channel blocker.

- 1 62. A method of claim 58 wherein the substance is a toxin.
- 1 63. A prescreening method for identifying potential modulators of potassium ion
2 channel function comprising:
 - 3 (a) binding a soluble potassium ion channel protein to a solid support where the ion
4 channel has the scaffold of a two-transmembrane-domain-type potassium ion
5 channel and has a tetrameric confirmation;
 - 6 (b) contacting the soluble potassium ion channel protein of step i with a compound in
7 an aqueous solution; and,
 - 8 (c) determining the binding of the compound to the soluble potassium ion channel
9 protein.
- 1 64. A method of claim 63 wherein the contacting takes place in the presence of a
2 detergent.
- 1 65. A method of claim 63 wherein the ion channel can pass potassium ions when
2 expressed in a cell.
- 1 66. A method of claim 63 which further comprises the contacting of the compound to
2 cell expressing a two-transmembrane-domain-type potassium ion channel protein
3 said cell cultured in an aqueous media containing potassium and determining
4 modulation of potassium flow between the inside of the cell and the media.
- 1 67. A column comprising a solid support having bound thereto an ion channel having
2 the scaffold of a two-transmembrane-domain-type potassium ion channel and having
3 a tetrameric confirmation.
- 1 68. A column of claim 25 wherein the ion channel is a non-natural and functional
2 two-transmembrane-domain-type potassium ion channel protein wherein the

3 non-natural protein is mutated in its amino acid sequence from a corresponding
4 natural protein whereby the mutation does not prevent the non-natural protein from
5 binding agitoxin2 when the non-natural protein is further mutated to correspond to
6 the agitoxin2 docking site of a Shaker K⁺ channel protein said docking site created
7 by substituting amino acid residues selected from within the 36 amino acid domain
8 defined by -25 to +5 of the Shaker K⁺ selectivity filter where the 0 residue is
9 either the phenylalanine or the tyrosine of the filter's signature sequence selected
10 from the group consisting of glycine-phenylalanine-glycine or
11 glycine-tyrosine-glycine.

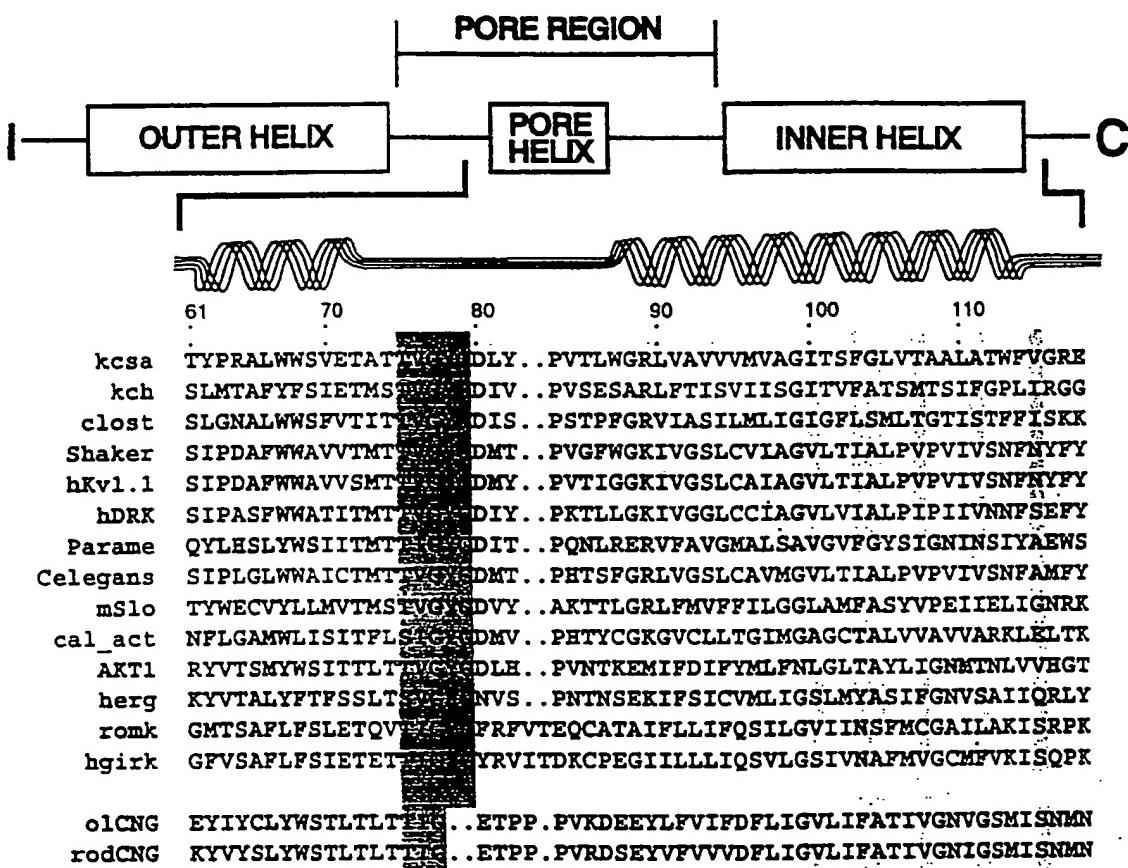


Figure 1

Figure 2(a)

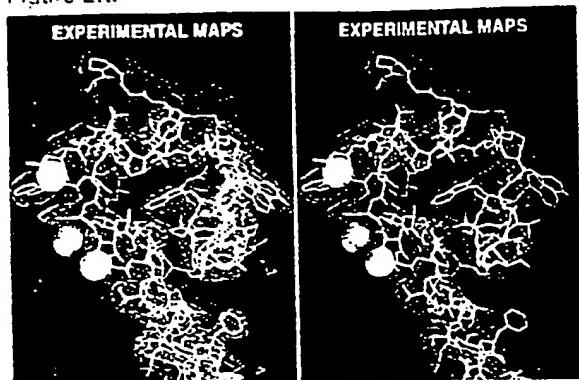


Figure 2(b)

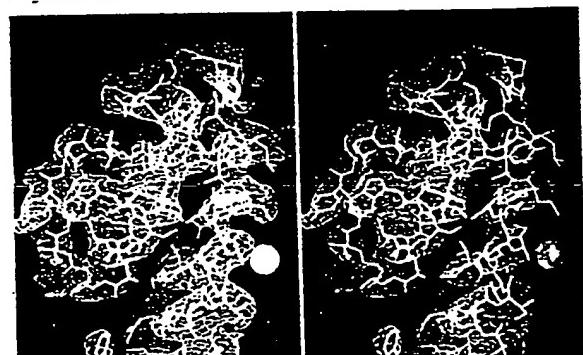


Figure 2(c)

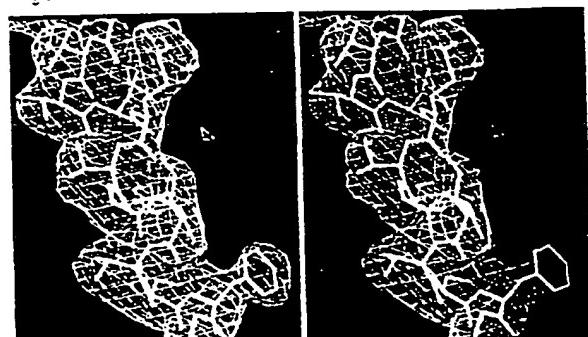


Figure 2

Figure 3(a).

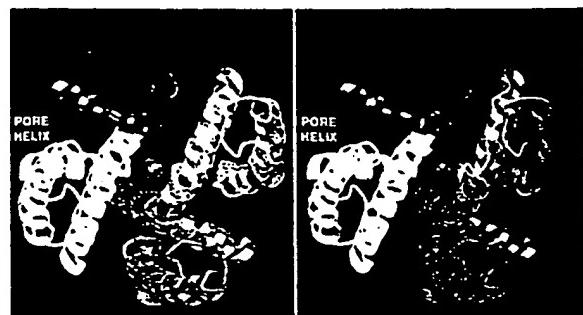


Figure 3(b).

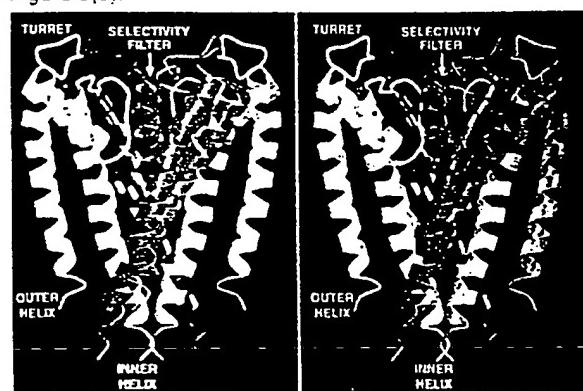


Figure 3(c)

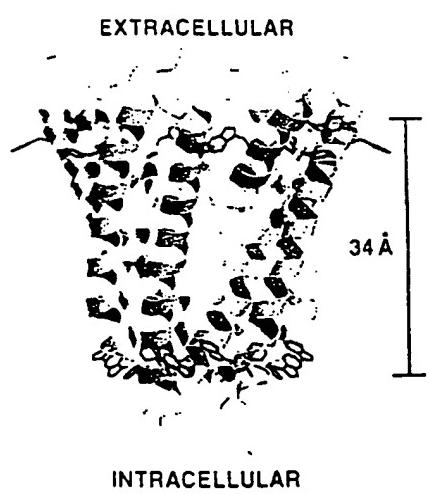


Figure 3

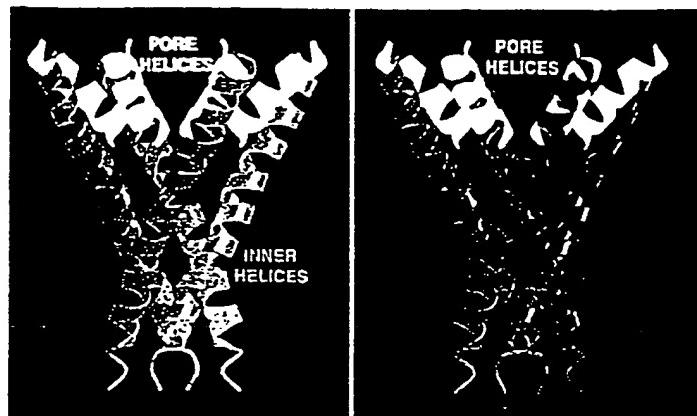


Figure 2

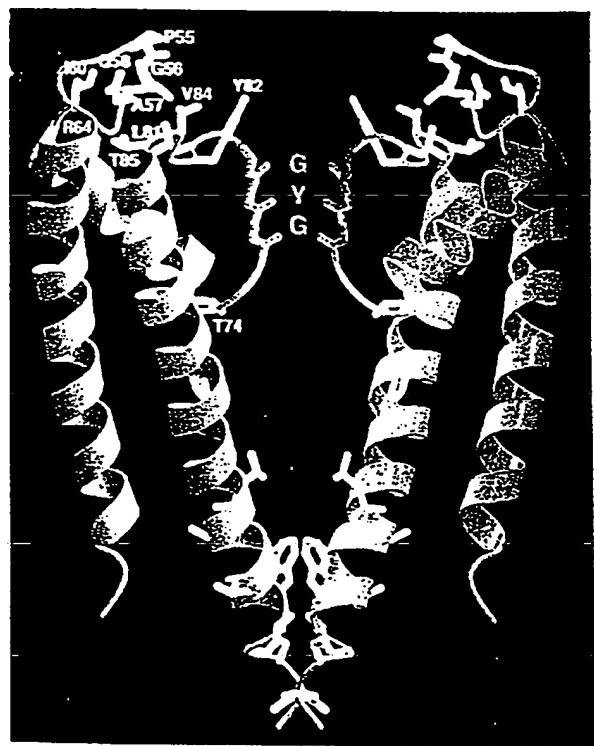


Figure 4

Figure 5(a).



Figure 5(b).

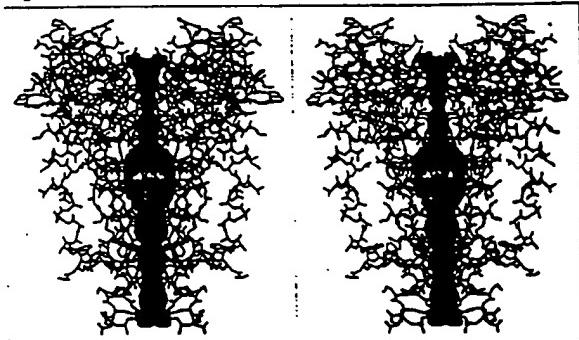
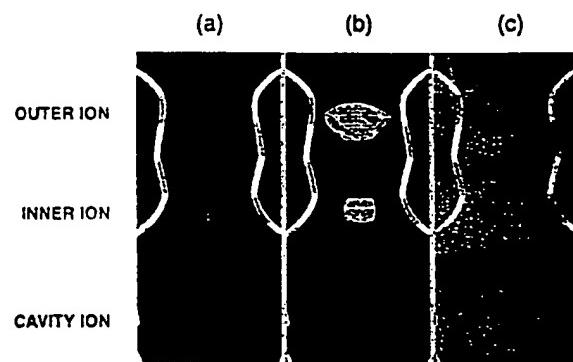


Figure 5

Figure 6.**Figure 6**

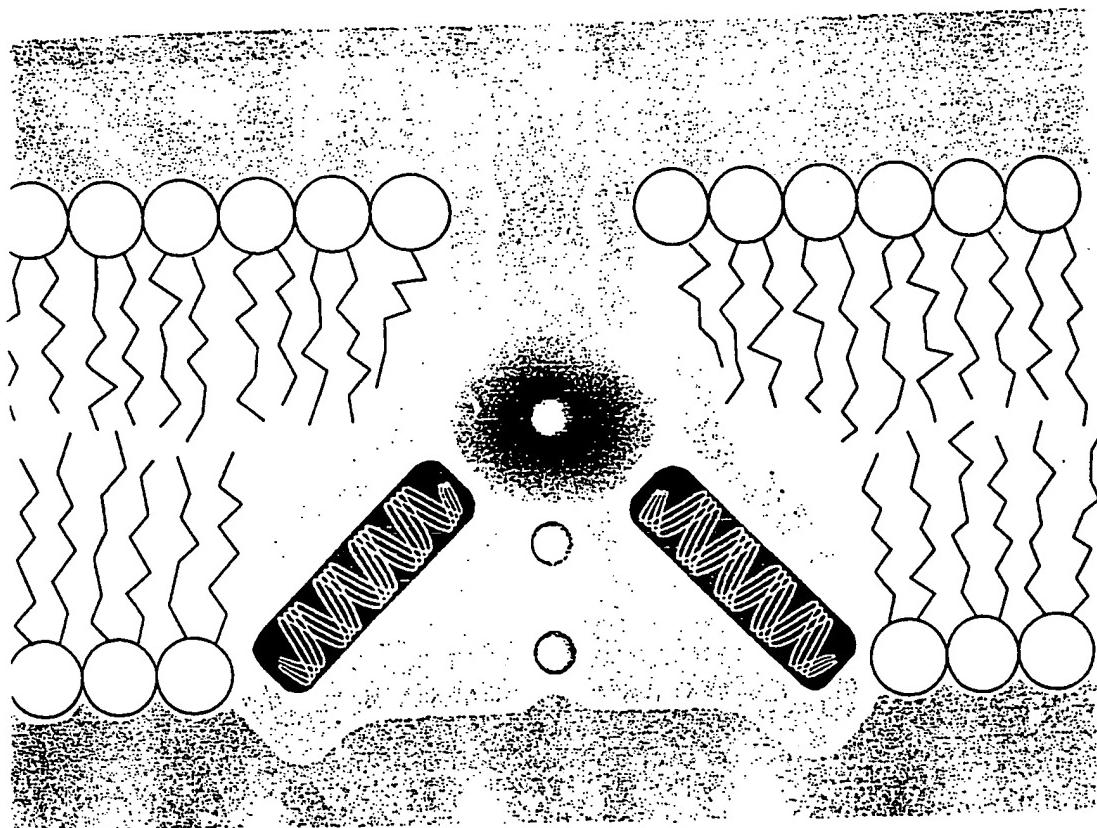


Figure 7

Figure 8(a).

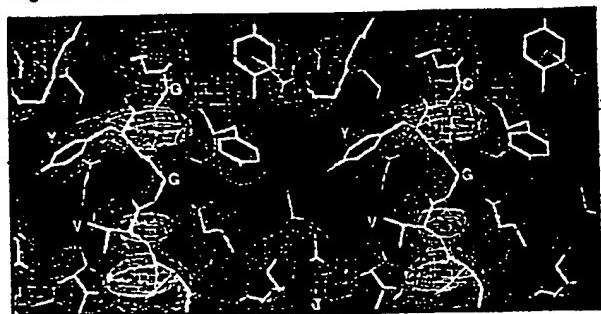


Figure 8(b).



Figure 8(c).

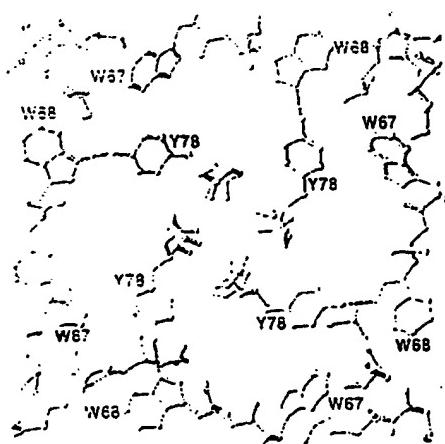


Figure 8

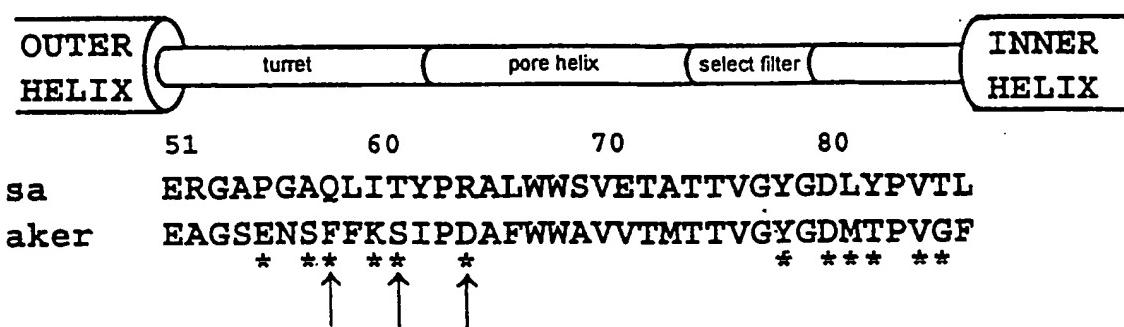
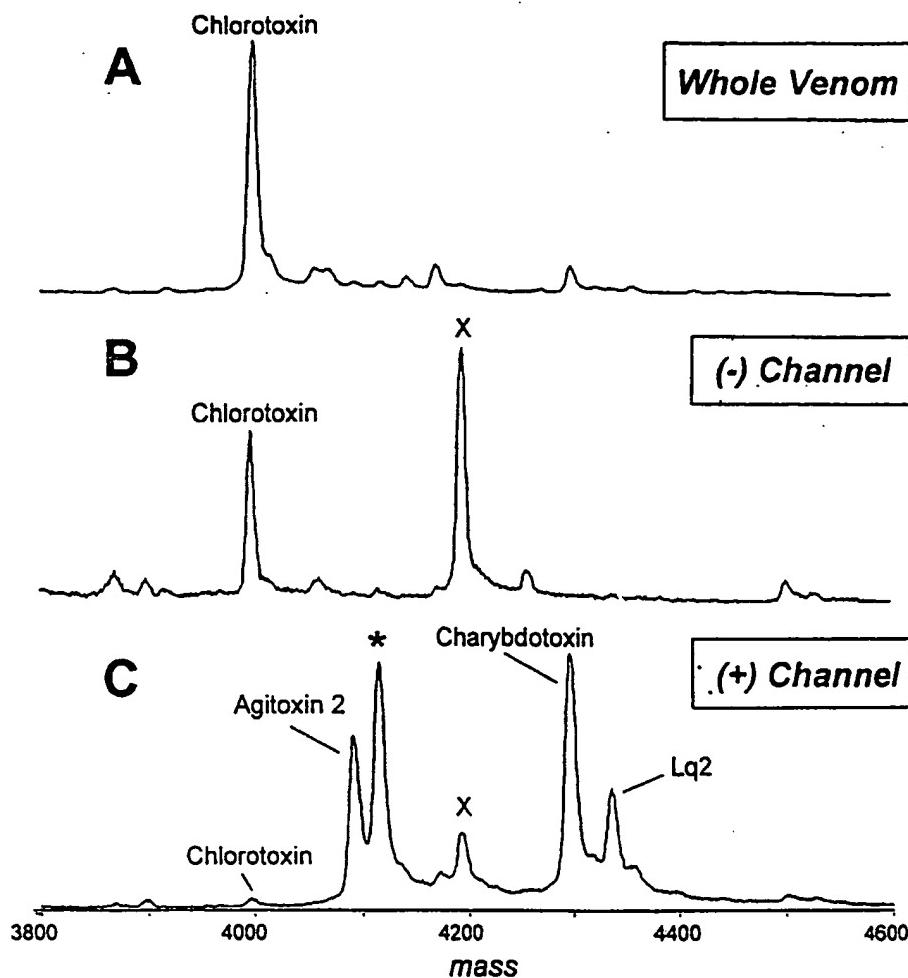


Figure 9

**D**

Toxin	Measured	Mass (Da) Calculated
Chlorotoxin	3996.4	3996.7
Agitoxin 2	4090.8	4090.9
*	4113.5	_____
Charybdotoxin	4295.9	4296.0
Lq2	4336.1	4336.0

Figure 10

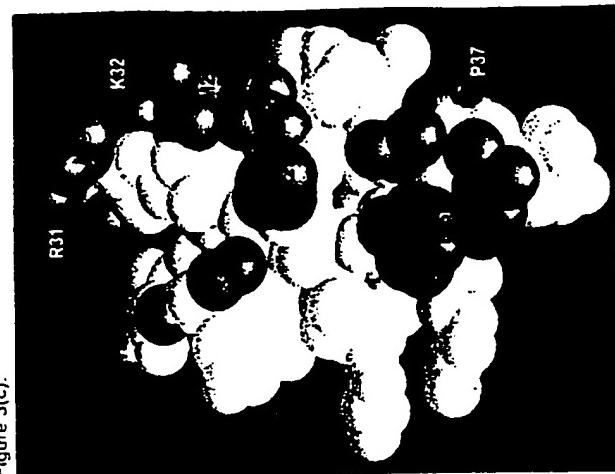
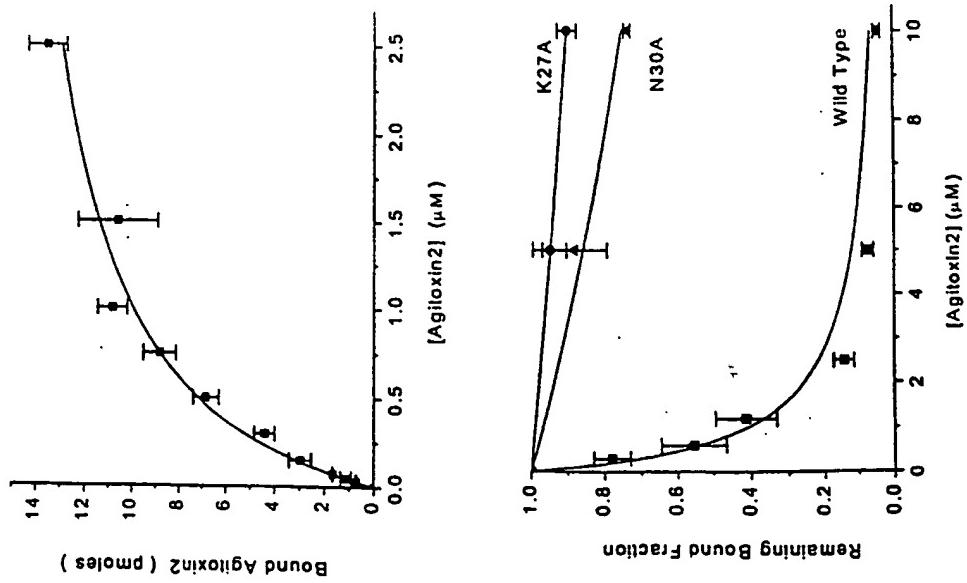


Figure 3(c).



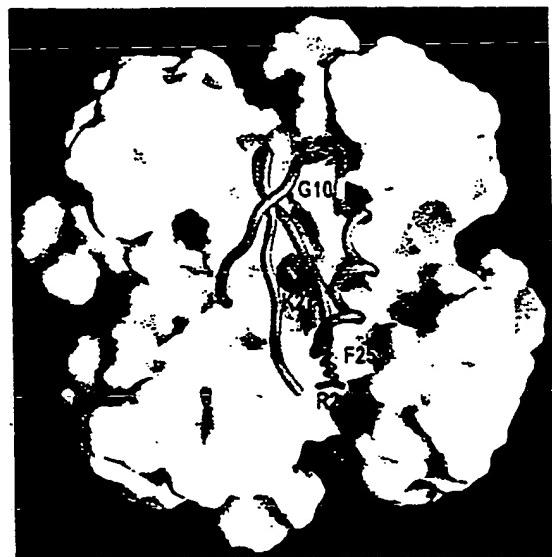
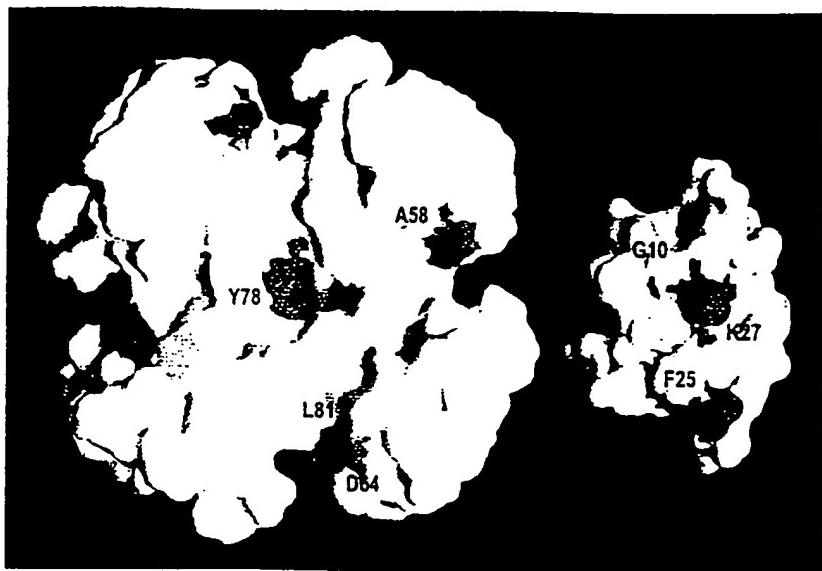


Figure 12

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: MacKinnon, Roderick
- (ii) TITLE OF INVENTION: ASSAYS FOR SCREENING COMPOUNDS WHICH INTERACT WITH CATION CHANNEL PROTEINS, MUTANT PROKARYOTIC CATION CHANNEL PROTEINS, AND USES THEREOF
- (iii) NUMBER OF SEQUENCES: 19
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Klauber & Jackson
 - (B) STREET: 411 Hackensack Avenue, 4th Floor
 - (C) CITY: Hackensack
 - (D) STATE: New Jersey
 - (E) COUNTRY: USA
 - (F) ZIP: 07601
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Jackson Esq., David A.
 - (B) REGISTRATION NUMBER: 26,742
 - (C) REFERENCE/DOCKET NUMBER: 600-1-220 CIP
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 201-487-5800
 - (B) TELEFAX: 201-343-1684
 - (C) TELEX: 133521

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 160 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Pro Pro Met Leu Ser Gly Leu Leu Ala Arg Leu Val Lys Leu Leu
1 5 10 15

Leu Gly Arg His Gly Ser Ala Leu His Trp Arg Ala Ala Gly Ala Ala

20

25

30

Thr Val Leu Leu Val Ile Val Leu Leu Ala Gly Ser Tyr Leu Ala Val		
35	40	45
Leu Ala Glu Arg Gly Ala Pro Gly Ala Gln Leu Ile Thr Tyr Pro Arg		
50	55	60
Ala Leu Trp Trp Ser Val Glu Thr Ala Thr Thr Val Gly Tyr Gly Asp		
65	70	75
Leu Tyr Pro Val Thr Leu Trp Gly Arg Leu Val Ala Val Val Met		
85	90	95
Val Ala Gly Ile Thr Ser Phe Gly Leu Val Thr Ala Ala Leu Ala Thr		
100	105	110
Trp Phe Val Gly Arg Glu Gln Glu Arg Arg Gly His Phe Val Arg His		
115	120	125
Ser Glu Lys Ala Ala Glu Glu Ala Tyr Thr Arg Thr Thr Arg Ala Leu		
130	135	140
His Glu Arg Phe Asp Arg Leu Glu Arg Met Leu Asp Asp Asn Arg Arg		
145	150	155
160		

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 417 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ser His Trp Thr Thr Phe Lys Gln Thr Ala Thr Lys Leu Trp Val		
1	5	10
15		
Thr Leu Arg His Asp Ile Leu Ala Leu Val Phe Leu Asn Gly Leu		
20	25	30
Leu Ile Phe Lys Thr Ile Tyr Gly Met Ser Val Asn Leu Leu Asp Ile		
35	40	45
Phe His Ile Lys Ala Phe Ser Glu Leu Asp Leu Ser Leu Leu Ala Asn		
50	55	60
Ala Pro Leu Phe Met Leu Gly Val Phe Leu Val Leu Asn Ser Ile Gly		
65	70	75
80		
Leu Leu Phe Arg Ala Lys Leu Ala Trp Ala Ile Ser Ile Ile Leu Leu		
85	90	95
Leu Ile Ala Leu Ile Tyr Thr Leu His Phe Tyr Pro Trp Leu Lys Phe		
100	105	110

Ser Ile Gly Phe Cys Ile Phe Thr Leu Val Phe Leu Leu Ile Leu Arg
 115 120 125
 Lys Asp Phe Ser His Ser Ser Ala Ala Ala Gly Thr Ile Phe Ala Phe
 130 135 140
 Ile Ser Phe Thr Thr Leu Leu Phe Tyr Ser Thr Tyr Gly Ala Leu Tyr
 145 150 155 160
 Leu Ser Glu Gly Phe Asn Pro Arg Ile Glu Ser Leu Met Thr Ala Phe
 165 170 175
 Tyr Phe Ser Ile Glu Thr Met Ser Thr Val Gly Tyr Gly Asp Ile Val
 180 185 190
 Pro Val Ser Glu Ser Ala Arg Leu Phe Thr Ile Ser Val Ile Ile Ser
 195 200 205
 Gly Ile Thr Val Phe Ala Thr Ser Met Thr Ser Ile Phe Gly Pro Leu
 210 215 220
 Ile Arg Gly Gly Phe Asn Lys Leu Val Lys Gly Asn Asn His Thr Met
 225 230 235 240
 His Arg Lys Asp His Phe Ile Val Cys Gly His Ser Ile Leu Ala Ile
 245 250 255
 Asn Thr Ile Leu Gln Leu Asn Gln Arg Gly Gln Asn Val Thr Val Ile
 260 265 270
 Ser Asn Leu Pro Glu Asp Asp Ile Lys Gln Leu Glu Gln Arg Leu Gly
 275 280 285
 Asp Asn Ala Asp Val Ile Pro Gly Asp Ser Asn Asp Ser Ser Val Leu
 290 295 300
 Lys Lys Ala Gly Ile Asp Arg Cys Arg Ala Ile Leu Ala Leu Ser Asp
 305 310 315 320
 Asn Asp Ala Asp Asn Ala Phe Val Val Leu Ser Ala Lys Asp Met Ser
 325 330 335
 Ser Asp Val Lys Thr Val Leu Ala Val Ser Asp Ser Lys Asn Leu Asn
 340 345 350
 Lys Ile Lys Met Val His Pro Asp Ile Ile Leu Ser Pro Gln Leu Phe
 355 360 365
 Gly Ser Glu Ile Leu Ala Arg Val Leu Asn Gly Glu Glu Ile Asn Asn
 370 375 380
 Asp Met Leu Val Ser Met Leu Leu Asn Ser Gly His Gly Ile Phe Ser
 385 390 395 400
 Asp Asn Asp Glu Gln Glu Thr Lys Ala Asp Ser Lys Glu Ser Ala Gln
 405 410 415
 Lys

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 58 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ser	Leu	Gly	Asn	Ala	Leu	Trp	Trp	Ser	Phe	Val	Thr	Ile	Thr	Thr	Val
1					5					10					15
Gly	Tyr	Gly	Asp	Ile	Ser	Pro	Ser	Thr	Pro	Phe	Gly	Arg	Val	Ile	Ala
				20				25				30			
Ser	Ile	Leu	Met	Leu	Ile	Gly	Ile	Gly	Phe	Leu	Ser	Met	Leu	Thr	Gly
			35				40					45			
Thr	Ile	Ser	Thr	Phe	Phe	Ile	Ser	Lys	Lys						
			50				55								

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 616 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met	Ala	Ala	Val	Ala	Gly	Leu	Tyr	Gly	Leu	Gly	Glu	Asp	Arg	Gln	His
1				5				10						15	
Arg	Lys	Lys	Gln	Gln	Gln	Gln	Gln	His	Gln	Lys	Glu	Gln	Leu	Glu	
			20				25					30			
Gln	Lys	Glu	Gln	Gln	Lys	Ile	Ala	Glu	Arg	Lys	Leu	Gln	Leu	Arg	
			35			40						45			
Glu	Gln	Gln	Leu	Gln	Arg	Asn	Ser	Leu	Asp	Gly	Tyr	Gly	Ser	Leu	Pro
			50			55		60							
Lys	Leu	Ser	Ser	Gln	Asp	Glu	Glu	Gly	Gly	Ala	Gly	His	Gly	Phe	Gly
			65		70			75					80		
Gly	Gly	Pro	Gln	His	Phe	Glu	Pro	Ile	Pro	His	Asp	His	Asp	Phe	Cys
			85				90					95			
Glu	Arg	Val	Val	Ile	Asn	Val	Ser	Gly	Leu	Arg	Phe	Glu	Thr	Gln	Leu
			100			105						110			

Arg Thr Leu Asn Gln Phe Pro Asp Thr Leu Leu Gly Asp Pro Ala Arg
 115 120 125

Arg Leu Arg Tyr Phe Asp Pro Leu Arg Asn Glu Tyr Phe Phe Asp Arg
 130 135 140

Ser Arg Pro Ser Phe Asp Ala Ile Leu Tyr Tyr Tyr Gln Ser Gly Gly
 145 150 155 160

Arg Leu Arg Arg Pro Val Asn Val Pro Leu Asp Val Phe Ser Glu Glu
 165 170 175

Ile Lys Phe Tyr Glu Leu Gly Asp Gln Ala Ile Asn Lys Phe Arg Glu
 180 185 190

Asp Glu Gly Phe Ile Lys Glu Glu Glu Arg Pro Leu Pro Asp Asn Glu
 195 200 205

Lys Gln Arg Lys Val Trp Leu Leu Phe Glu Tyr Pro Glu Ser Ser Gln
 210 215 220

Ala Ala Arg Val Val Ala Ile Ile Ser Val Phe Val Ile Leu Leu Ser
 225 230 235 240

Ile Val Ile Phe Cys Leu Glu Thr Leu Pro Glu Phe Lys His Tyr Lys
 245 250 255

Val Phe Asn Thr Thr Asn Gly Thr Lys Ile Glu Glu Asp Glu Val
 260 265 270

Pro Asp Ile Thr Asp Pro Phe Phe Leu Ile Gln Thr Leu Cys Ile Ile
 275 280 285

Trp Phe Thr Phe Glu Leu Thr Val Arg Phe Leu Ala Cys Pro Asn Lys
 290 295 300

Leu Asn Phe Cys Arg Asp Val Met Asn Val Ile Asp Ile Ile Ala Ile
 305 310 315 320

Ile Pro Tyr Phe Ile Thr Leu Ala Thr Val Val Ala Glu Glu Asp
 325 330 335

Thr Leu Asn Leu Pro Lys Ala Pro Val Ser Pro Gln Asp Lys Ser Ser
 340 345 350

Asn Gln Ala Met Ser Leu Ala Ile Leu Arg Val Ile Arg Leu Val Arg
 355 360 365

Val Phe Arg Ile Phe Lys Leu Ser Arg His Ser Lys Gly Leu Gln Ile
 370 375 380

Leu Gly Arg Thr Leu Lys Ala Ser Met Arg Glu Leu Gly Leu Leu Ile
 385 390 395 400

Phe Phe Leu Phe Ile Gly Val Val Leu Phe Ser Ser Ala Val Tyr Phe
 405 410 415

Ala Glu Ala Gly Ser Glu Asn Ser Phe Phe Lys Ser Ile Pro Asp Ala
 420 425 430

Phe Trp Trp Ala Val Val Thr Met Thr Thr Val Gly Tyr Gly Asp Met
 435 440 445

Thr Pro Val Gly Phe Trp Gly Lys Ile Val Gly Ser Leu Cys Val Ile
 450 455 460
 Ala Gly Val Leu Thr Ile Ala Leu Pro Val Pro Val Ile Val Ser Asn
 465 470 475 480
 Phe Asn Tyr Phe Tyr His Arg Glu Ala Asp Arg Glu Glu Met Gln Ser
 485 490 495
 Gln Asn Phe Asn His Val Thr Ser Cys Ser Tyr Leu Pro Gly Ala Leu
 500 505 510
 Gly Gln His Leu Lys Lys Ser Ser Leu Ser Glu Ser Ser Ser Asp Ile
 515 520 525
 Met Asp Leu Asp Asp Gly Ile Asp Ala Thr Thr Pro Gly Leu Thr Asp
 530 535 540
 His Thr Gly Arg His Met Val Pro Phe Leu Arg Thr Gln Gln Ser Phe
 545 550 555 560
 Glu Lys Gln Gln Leu Gln Leu Gln Leu Gln Gln Gln Gln Ser Gln
 565 570 575
 Ser Pro His Gly Gln Gln Met Thr Gln Gln Gln Leu Gly Gln Asn
 580 585 590
 Gly Leu Arg Ser Thr Asn Ser Leu Gln Leu Arg His Asn Asn Ala Met
 595 600 605
 Ala Val Ser Ile Glu Thr Asp Val
 610 615

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 495 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Thr Val Met Ser Gly Glu Asn Val Asp Glu Ala Ser Ala Ala Pro
 1 5 10 15
 Gly His Pro Gln Asp Gly Ser Tyr Pro Arg Gln Ala Asp His Asp Asp
 20 25 30
 His Glu Cys Cys Glu Arg Val Val Ile Asn Ile Ser Gly Leu Arg Phe
 35 40 45
 Glu Thr Gln Leu Lys Thr Leu Ala Gln Phe Pro Asn Thr Leu Leu Gly
 50 55 60

Asn	Pro	Lys	Lys	Arg	Met	Arg	Tyr	Phe	Asp	Pro	Leu	Arg	Asn	Glu	Tyr
65					70					75				80	
Phe	Phe	Asp	Arg	Asn	Arg	Pro	Ser	Phe	Asp	Ala	Ile	Leu	Tyr	Tyr	Tyr
						85					90			95	
Gln	Ser	Gly	Gly	Arg	Leu	Arg	Arg	Pro	Val	Asn	Val	Pro	Leu	Asp	Met
					100				105			110			
Phe	Ser	Glu	Glu	Ile	Lys	Phe	Tyr	Glu	Leu	Gly	Glu	Glu	Ala	Met	Glu
					115				120			125			
Lys	Phe	Arg	Glu	Asp	Glu	Gly	Phe	Ile	Lys	Glu	Glu	Glu	Arg	Pro	Leu
					130			135			140				
Pro	Glu	Lys	Glu	Tyr	Gln	Arg	Gln	Val	Trp	Leu	Leu	Phe	Glu	Tyr	Pro
					145			150			155			160	
Glu	Ser	Ser	Gly	Pro	Ala	Arg	Val	Ile	Ala	Ile	Val	Ser	Val	Met	Val
					165			170			175				
Ile	Leu	Ile	Ser	Ile	Val	Ile	Phe	Cys	Leu	Glu	Thr	Leu	Pro	Glu	Leu
					180			185			190				
Lys	Asp	Asp	Lys	Asp	Phe	Thr	Gly	Thr	Val	His	Arg	Ile	Asp	Asn	Thr
					195			200			205				
Thr	Val	Ile	Tyr	Asn	Ser	Asn	Ile	Phe	Thr	Asp	Pro	Phe	Phe	Ile	Val
					210			215			220				
Glu	Thr	Leu	Cys	Ile	Ile	Trp	Phe	Ser	Phe	Glu	Leu	Val	Val	Arg	Phe
					225			230			235			240	
Phe	Ala	Cys	Pro	Ser	Lys	Thr	Asp	Phe	Phe	Lys	Asn	Ile	Met	Asn	Phe
					245			250			255				
Ile	Asp	Ile	Val	Ala	Ile	Ile	Pro	Tyr	Phe	Ile	Thr	Leu	Gly	Thr	Glu
					260			265			270				
Ile	Ala	Glu	Gln	Glu	Gly	Asn	Gln	Lys	Gly	Glu	Gln	Ala	Thr	Ser	Leu
					275			280			285				
Ala	Ile	Leu	Arg	Val	Ile	Arg	Leu	Val	Arg	Val	Phe	Arg	Ile	Phe	Lys
					290			295			300				
Leu	Ser	Arg	His	Ser	Lys	Gly	Leu	Gln	Ile	Leu	Gly	Gln	Thr	Leu	Lys
					305			310			315			320	
Ala	Ser	Met	Arg	Glu	Leu	Gly	Leu	Leu	Ile	Phe	Phe	Leu	Phe	Ile	Gly
					325			330			335				
Val	Ile	Leu	Phe	Ser	Ser	Ala	Val	Tyr	Phe	Ala	Glu	Ala	Glu	Glu	Ala
					340			345			350				
Glu	Ser	His	Phe	Ser	Ser	Ile	Pro	Asp	Ala	Phe	Trp	Trp	Ala	Val	Val
					355			360			365				
Ser	Met	Thr	Thr	Val	Gly	Tyr	Gly	Asp	Met	Tyr	Pro	Val	Thr	Ile	Gly
					370			375			380				
Gly	Lys	Ile	Val	Gly	Ser	Leu	Cys	Ala	Ile	Ala	Gly	Val	Leu	Thr	Ile
					385			390			395			400	

Ala Leu Pro Val Pro Val Ile Val Ser Asn Phe Asn Tyr Phe Tyr His
 405 410 415
 Arg Glu Thr Glu Gly Glu Gln Ala Gln Leu Leu His Val Ser Ser
 420 425 430
 Pro Asn Leu Ala Ser Asp Ser Asp Leu Ser Arg Arg Ser Ser Ser Thr
 435 440 445
 Met Ser Lys Tyr Glu Tyr Met Glu Ile Glu Glu Asp Met Asn Asn Ser
 450 455 460
 Ile Ala His Tyr Arg Gln Val Asn Ile Arg Thr Ala Asn Cys Thr Thr
 465 470 475 480
 Ala Asn Gln Asn Cys Val Asn Lys Ser Lys Leu Leu Thr Asp Val
 485 490 495

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 858 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Pro Ala Gly Met Thr Lys His Gly Ser Arg Ser Thr Ser Ser Leu
 1 5 10 15
 Pro Pro Glu Pro Met Glu Ile Val Arg Ser Lys Ala Cys Ser Arg Arg
 20 25 30
 Val Arg Leu Asn Val Gly Gly Leu Ala His Glu Val Leu Trp Arg Thr
 35 40 45
 Leu Asp Arg Leu Pro Arg Thr Arg Leu Gly Lys Leu Arg Asp Cys Asn
 50 55 60
 Thr His Asp Ser Leu Leu Glu Val Cys Asp Asp Tyr Ser Leu Asp Asp
 65 70 75 80
 Asn Glu Tyr Phe Phe Asp Arg His Pro Gly Ala Phe Thr Ser Ile Leu
 85 90 95
 Asn Phe Tyr Arg Thr Gly Arg Leu His Met Met Glu Glu Met Cys Ala
 100 105 110
 Leu Ser Phe Ser Gln Glu Leu Asp Tyr Trp Gly Ile Asp Glu Ile Tyr
 115 120 125
 Leu Glu Ser Cys Cys Gln Ala Arg Tyr His Gln Lys Lys Glu Gln Met
 130 135 140
 Asn Glu Glu Leu Lys Arg Glu Ala Glu Thr Leu Arg Glu Arg Glu Gly

145	150	155	160
Glu Glu Phe Asp Asn Thr Cys Cys Ala Glu Lys Arg Lys Lys Leu Trp			
165		170	175
Asp Leu Leu Glu Lys Pro Asn Ser Ser Val Ala Ala Lys Ile Leu Ala			
180		185	190
Ile Ile Ser Ile Met Phe Ile Val Leu Ser Thr Ile Ala Leu Ser Leu			
195		200	205
Asn Thr Leu Pro Glu Leu Gln Ser Leu Asp Glu Phe Gly Gln Ser Thr			
210		215	220
Asp Asn Pro Gln Leu Ala His Val Glu Ala Val Cys Ile Ala Trp Phe			
225		230	235
Thr Met Glu Tyr Leu Leu Arg Phe Leu Ser Ser Pro Lys Lys Trp Lys			
245		250	255
Phe Phe Lys Gly Pro Leu Asn Ala Ile Asp Leu Leu Ala Ile Leu Pro			
260		265	270
Tyr Tyr Val Thr Ile Phe Leu Thr Glu Ser Asn Lys Ser Val Leu Gln			
275		280	285
Phe Gln Asn Val Arg Arg Val Val Gln Ile Phe Arg Ile Met Arg Ile			
290		295	300
Leu Arg Ile Leu Lys Leu Ala Arg His Ser Thr Gly Leu Gln Ser Leu			
305		310	315
Gly Phe Thr Leu Arg Arg Ser Tyr Asn Glu Leu Gly Leu Leu Ile Leu			
325		330	335
Phe Leu Ala Met Gly Ile Met Ile Phe Ser Ser Leu Val Phe Phe Ala			
340		345	350
Glu Lys Asp Glu Asp Asp Thr Lys Phe Lys Ser Ile Pro Ala Ser Phe			
355		360	365
Trp Trp Ala Thr Ile Thr Met Thr Thr Val Gly Tyr Gly Asp Ile Tyr			
370		375	380
Pro Lys Thr Leu Leu Gly Lys Ile Val Gly Gly Leu Cys Cys Ile Ala			
385		390	395
Gly Val Leu Val Ile Ala Leu Pro Ile Pro Ile Ile Val Asn Asn Phe			
405		410	415
Ser Glu Phe Tyr Lys Glu Gln Lys Arg Gln Glu Lys Ala Ile Lys Arg			
420		425	430
Arg Glu Ala Leu Glu Arg Ala Lys Arg Asn Gly Ser Ile Val Ser Met			
435		440	445
Asn Met Lys Asp Ala Phe Ala Arg Ser Ile Glu Met Met Asp Ile Val			
450		455	460
Val Glu Lys Asn Gly Glu Asn Met Gly Lys Lys Asp Lys Val Gln Asp			
465		470	475
Asn His Leu Ser Pro Asn Lys Trp Lys Trp Thr Lys Arg Thr Leu Ser			

485	490	495
Glu Thr Ser Ser Ser Lys Ser Phe Glu Thr Lys Glu Gln Gly Ser Pro		
500	505	510
Glu Lys Ala Arg Ser Ser Ser Pro Gln His Leu Asn Val Gln Gln		
515	520	525
Leu Glu Asp Met Tyr Asn Lys Met Ala Lys Thr Gln Ser Gln Pro Ile		
530	535	540
Leu Asn Thr Lys Glu Ser Ala Ala Gln Ser Lys Pro Lys Glu Glu Leu		
545	550	555
560		
Glu Met Glu Ser Ile Pro Ser Pro Val Ala Pro Leu Pro Thr Arg Thr		
565	570	575
Glu Gly Val Ile Asp Met Arg Ser Met Ser Ser Ile Asp Ser Phe Ile		
580	585	590
Ser Cys Ala Thr Asp Phe Pro Glu Ala Thr Arg Phe Ser His Ser Pro		
595	600	605
Leu Thr Ser Leu Pro Ser Lys Thr Gly Gly Ser Thr Ala Pro Glu Val		
610	615	620
Gly Trp Arg Gly Ala Leu Gly Ala Ser Gly Gly Arg Phe Val Glu Ala		
625	630	635
640		
Asn Pro Ser Pro Asp Ala Ser Gln His Ser Ser Phe Phe Ile Glu Ser		
645	650	655
Pro Lys Ser Ser Met Lys Thr Asn Asn Pro Leu Lys Leu Arg Ala Leu		
660	665	670
Lys Val Asn Phe Met Glu Gly Asp Pro Ser Pro Leu Leu Pro Val Leu		
675	680	685
Gly Met Tyr His Asp Pro Leu Arg Asn Arg Gly Ser Ala Ala Ala Ala		
690	695	700
Val Ala Gly Leu Glu Cys Ala Thr Leu Leu Asp Lys Ala Val Leu Ser		
705	710	715
720		
Pro Glu Ser Ser Ile Tyr Thr Thr Ala Ser Ala Lys Thr Pro Pro Arg		
725	730	735
Ser Pro Glu Lys His Thr Ala Ile Ala Phe Asn Phe Glu Ala Gly Val		
740	745	750
His Gln Tyr Ile Asp Ala Asp Thr Asp Asp Glu Gly Gln Leu Leu Tyr		
755	760	765
Ser Val Asp Ser Ser Pro Pro Lys Ser Leu Pro Gly Ser Thr Ser Pro		
770	775	780
Lys Phe Ser Thr Gly Thr Arg Ser Glu Lys Asn His Phe Glu Ser Ser		
785	790	795
800		
Pro Leu Pro Thr Ser Pro Lys Phe Leu Arg Gln Asn Cys Ile Tyr Ser		
805	810	815
Thr Glu Ala Leu Thr Gly Lys Gly Pro Ser Gly Gln Glu Lys Cys Lys		

820	825	830
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Leu Glu Asn His Ile Ser Pro Asp Val Arg Val Leu Pro Gly Gly Gly	835	840	845
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Ala His Gly Ser Thr Arg Asp Gln Ser Ile	850	855
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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 597 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Gly Pro Lys Ile Arg Ala Ile Ser Phe Gln Ser Asn Lys Pro Met	1	5	10	15
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Met Asn Leu Lys Glu Asp Ser Pro Val Phe Ile Asp Ser His Thr Asp	20	25	30
---	----	----	----

His Val Gly Phe Ser Asn Lys Ile Trp Arg Thr Lys Ala Leu Glu Ile	35	40	45
---	----	----	----

Leu Met Ile Thr Leu Arg Phe Ile Ser Phe Ile Thr Lys Ser Asn Phe	50	55	60
---	----	----	----

Ala Thr Ser Phe Lys Leu Ile Asn Lys Asn Val Phe Glu Ile Ile Gly	65	70	75	80
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Asp Val Ser Ala Asp Phe Thr Tyr Tyr Leu Leu Lys Asn Phe Phe Lys	85	90	95
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Tyr Glu Lys Pro Thr Gly Phe Gln Lys Gly Gln His Phe Leu Asn Gln	100	105	110
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Thr Leu Leu Ile Pro Leu Arg Lys Thr Lys Leu Leu Lys Ile Tyr Cys	115	120	125
---	-----	-----	-----

Gly Asn Gln Lys Leu Ile Met Arg Pro Glu Ser Leu Ala Ser Ile Trp	130	135	140
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Trp Asn Ile Tyr Ile Leu Thr Ile Leu Asn Ile Asn Val Leu Tyr Val	145	150	155	160
---	-----	-----	-----	-----

Ser Ile Lys Ile Ala Phe Lys Phe Asp Glu Gln Ser Gln Asp Asp Phe	165	170	175
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Tyr Gln Ala Arg Gln Ile Ile Phe Asp Val Leu Pro Ser Tyr Ser Phe	180	185	190
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Met Leu Glu Ile Leu Leu Lys Phe Asn Thr Cys Tyr Tyr Lys Gly	195	200	205
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Ala Val Ile Glu Asn Arg Tyr Gln Ile Ala Lys Asn Tyr Leu Arg Ser
 210 215 220
 Ser Phe Phe Phe Asp Ile Phe Val Val Ile Pro Tyr Phe Ile Ser Leu
 225 230 235 240
 Arg Phe Asp Leu Gln Tyr Leu Asp Leu Val Ile Ile Leu Lys Val Phe
 245 250 255
 Gln Ile Thr Lys Phe Ser Arg Asn Leu Phe Asp Arg Leu Glu Leu Thr
 260 265 270
 Ala Ile Gln Ile Val Ile Val Asp Leu Val Lys Leu Gly Tyr Thr Ile
 275 280 285
 Leu Ala Ala Ala His Phe Ser Ala Cys Ile Trp Phe Leu Val Gly Ser
 290 295 300
 Thr Gly Asn Pro Asn Asp Thr Ser Trp Ile Lys Ala Gln Asn Ile Glu
 305 310 315 320
 Asn Glu Gln Trp Phe Asn Gln Tyr Leu His Ser Leu Tyr Trp Ser Ile
 325 330 335
 Ile Thr Met Thr Thr Ile Gly Tyr Gly Asp Ile Thr Pro Gln Asn Leu
 340 345 350
 Arg Glu Arg Val Phe Ala Val Gly Met Ala Leu Ser Ala Val Gly Val
 355 360 365
 Phe Gly Tyr Ser Ile Gly Asn Ile Asn Ser Ile Tyr Ala Glu Trp Ser
 370 375 380
 Arg Gln Ser Phe Gln Ile Arg Thr Asp Met Asn Asn Leu Lys Lys Phe
 385 390 395 400
 Ile Arg Ile Lys Gly Ile Asn Lys His Leu Ala Glu Lys Ile Arg Lys
 405 410 415
 Tyr Phe Glu Tyr Val Trp Ser Asp Gln Met Glu Asp Asn Asp Arg Glu
 420 425 430
 Val Tyr Lys Phe Ser Glu Met Ile Pro Lys Gln Leu Ala Glu Glu Met
 435 440 445
 Lys Ile Asp Thr Asn Met Lys Leu Ile Gln Lys Asn Ser Phe Leu Val
 450 455 460
 Asn Asn Phe Ser Glu Gln Phe Leu Ile Ser Leu Ser Lys Val Leu Ile
 465 470 475 480
 Glu Glu Lys Tyr Val Pro Glu Ser Thr Ile Tyr Leu Val Lys Leu Ile
 485 490 495
 Asn Ile Leu Gln Gln Asn Asp Pro Ser Asn Tyr Leu Tyr Ile Leu Ser
 500 505 510
 Asn Gly Ser Leu Ser Phe Tyr Ile Thr Leu Asn Asn Lys Gln Gln Thr
 515 520 525
 Ile Lys Val Leu Glu Thr Ile Lys Asn Glu Gly Gln Ala Phe Gly Val
 530 535 540

Leu Glu Phe Phe Gln Ser Gln Ala Tyr Gln Val Ser Cys Lys Ser Asn
 545 550 555 560
 Gln Phe Ser Tyr Val Leu Lys Ile Asp Lys Ser Gln Phe Met Glu Ile
 565 570 575
 Ile Ser Gln His Lys Asn Asp Tyr Val Thr Gln Ile Ile Tyr Leu Ile
 580 585 590
 Leu Val Gln Ile Leu
 595

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 556 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Asp Ala Cys Ser Phe Asn Arg Phe Asp Ser Asn Arg Ser Ser Ala Arg
 1 5 10 15
 Arg Phe Ser Arg Arg Gly Ser Asp Tyr Phe Gly Asp Lys Gly Ile Ser
 20 25 30
 Met Asp Glu Arg Ile Val Leu Asn Val Gly Gly Val Arg His Glu Thr
 35 40 45
 Tyr Gln Ala Thr Leu Lys Lys Ile Pro Ala Thr Arg Leu Ser Arg Leu
 50 55 60
 Thr Pro Ser Leu Ala Asn Phe Asp Pro Leu Leu Asn Glu Tyr Phe Phe
 65 70 75 80
 Asp Arg His Pro Ala Val Phe Ala Met Ile Leu Asn Tyr Tyr Arg Thr
 85 90 95
 Gly Lys Leu His Tyr Pro Thr Asp Val Cys Gly Pro Leu Phe Glu Glu
 100 105 110
 Glu Leu Gln Tyr Trp Gly Leu Asp Ala Ser Asp Thr Glu Pro Cys Cys
 115 120 125
 Trp Met Gln Leu Leu His Ala Lys Asp Thr Gln Glu Thr Leu Ala Val
 130 135 140
 Leu Asp Arg Met Asp Ala Asp His Glu Asp Asp Pro Gln Leu Arg Glu
 145 150 155 160
 Gln Asp Thr Met Lys Lys Phe Gly Trp Glu Glu Asp Tyr Phe Gln Gly
 165 170 175
 Lys Arg Thr Arg Trp Met Lys Leu Lys Pro Gln Met Trp Ser Leu Phe

180	185	190
Asp Glu Pro Tyr Ser Ser Gln Ala Ala Lys Leu Ile Ala Gly Ile Ser		
195	200	205
Val Leu Phe Ile Phe Ile Ser Ile Phe Ser Phe Cys Leu Lys Thr His		
210	215	220
Gln Ser Phe Arg Leu Pro Val Leu Ile Gly Gln Asn Ile Thr Met Pro		
225	230	235
Gly Gly Val Val Gln Pro Ser Ile Glu Arg Val Ser Thr Glu Pro Leu		
245	250	255
Pro Ile Phe Gly Gln Ile Glu Met Leu Cys Asn Ile Trp Phe Thr Leu		
260	265	270
Glu Leu Ile Ile Arg Phe Val Phe Cys Pro Ser Lys Ile Arg Phe Phe		
275	280	285
Lys Ser Pro Leu Asn Met Ile Asp Leu Val Ala Thr Leu Ser Phe Tyr		
290	295	300
Ala Asp Ala Met Met Val Arg Val Val Glu Asp Glu Pro Lys Asp Val		
305	310	315
Val Glu Phe Leu Ser Met Ile Arg Ile Phe Arg Leu Phe Lys Leu Thr		
325	330	335
Gln His His Gln Gly Leu Gln Ile Leu Ile His Thr Phe Arg Ala Ser		
340	345	350
Ala Lys Glu Leu Ile Leu Val Phe Phe Leu Ile Leu Gly Ile Val		
355	360	365
Ile Phe Ala Ala Leu Val Tyr Tyr Ala Glu Lys Met Glu Ala Asn Pro		
370	375	380
Asn Asn Gln Phe Gln Ser Ile Pro Leu Gly Leu Trp Trp Ala Ile Cys		
385	390	395
Thr Met Thr Thr Val Gly Tyr Gly Asp Met Thr Pro His Thr Ser Phe		
405	410	415
Gly Arg Leu Val Gly Ser Leu Cys Ala Val Met Gly Val Leu Thr Ile		
420	425	430
Ala Leu Pro Val Pro Val Ile Val Ser Asn Phe Ala Met Phe Tyr Ser		
435	440	445
His Asn Gln Ala Arg Asp Lys Leu Pro Lys Arg Arg Arg Arg Val Leu		
450	455	460
Pro Val Glu Gln Ile Arg Leu Gln Ala Arg Arg His Ala Ala Val Leu		
465	470	475
Glu Pro Ser Ala Ser Gln Gly Gly Leu Gly Gly Gly Gln Ala Ile Arg		
485	490	495
Arg Arg Asn Met Pro Ile Leu Ile Asp Gln Asn Cys Cys Asp Glu Glu		
500	505	510
Asn His Asn His Lys Asp Arg Glu Lys Ser Glu Asn Ser Asp Glu Gly		

515

520

525

Thr Asn Ser Ser Ser Thr Thr Gly Val Asp Thr Val Val Lys Leu Gly
 530 535 540

Pro Ser Glu Thr Ala Ile Thr Thr Thr Ile Ile Ser
 545 550 555

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1196 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Glu Leu Glu His Pro Lys Ser Pro Pro Tyr Pro Ser Ser Ser Ser
 1 5 10 15

Ser Ser Ser Ser Ser Ser Val His Glu Pro Lys Met Asp Ala Leu Ile
 20 25 30

Ile Pro Val Thr Met Glu Val Pro Cys Asp Ser Arg Gly Gln Arg Met
 35 40 45

Trp Trp Ala Phe Leu Ala Ser Ser Met Val Thr Phe Phe Gly Gly Leu
 50 55 60

Phe Ile Ile Leu Leu Trp Arg Thr Leu Lys Tyr Leu Trp Thr Val Cys
 65 70 75 80

Cys His Cys Gly Gly Lys Thr Lys Glu Ala Gln Lys Ile Asn Asn Gly
 85 90 95

Ser Ser Gln Ala Asp Gly Thr Leu Lys Pro Val Asp Glu Lys Glu Glu
 100 105 110

Val Val Ala Ala Glu Val Gly Trp Met Thr Ser Val Lys Asp Trp Ala
 115 120 125

Gly Val Met Ile Ser Ala Gln Thr Leu Thr Gly Arg Val Leu Val Val
 130 135 140

Leu Val Phe Ala Leu Ser Ile Gly Ala Leu Val Ile Tyr Phe Ile Asp
 145 150 155 160

Ser Ser Asn Pro Ile Glu Ser Cys Gln Asn Phe Tyr Lys Asp Phe Thr
 165 170 175

Leu Gln Ile Asp Met Ala Phe Asn Val Phe Phe Leu Leu Tyr Phe Gly
 180 185 190

Leu Arg Phe Ile Ala Ala Asn Asp Lys Leu Trp Phe Trp Leu Glu Val
 195 200 205

Asn Ser Val Val Asp Phe Phe Thr Val Pro Pro Val Phe Val Ser Val
 210 215 220
 Tyr Leu Asn Arg Ser Trp Leu Gly Leu Arg Phe Leu Arg Ala Leu Arg
 225 230 235 240
 Leu Ile Gln Phe Ser Glu Ile Leu Gln Phe Leu Asn Ile Leu Lys Thr
 245 250 255
 Ser Asn Ser Ile Lys Leu Val Asn Leu Leu Ser Ile Phe Ile Ser Thr
 260 265 270
 Trp Leu Thr Ala Ala Gly Phe Ile His Leu Val Glu Asn Ser Gly Asp
 275 280 285
 Pro Trp Glu Asn Phe Gln Asn Asn Gln Ala Leu Thr Tyr Trp Glu Cys
 290 295 300
 Val Tyr Leu Leu Met Val Thr Met Ser Thr Val Gly Tyr Gly Asp Val
 305 310 315 320
 Tyr Ala Lys Thr Thr Leu Gly Arg Leu Phe Met Val Phe Phe Ile Leu
 325 330 335
 Gly Gly Leu Ala Met Phe Ala Ser Tyr Val Pro Glu Ile Ile Glu Leu
 340 345 350
 Ile Gly Asn Arg Lys Lys Tyr Gly Ser Tyr Ser Ala Val Ser Gly
 355 360 365
 Arg Lys His Ile Val Val Cys Gly His Ile Thr Leu Glu Ser Val Ser
 370 375 380
 Asn Phe Leu Lys Asp Phe Leu His Lys Asp Arg Asp Asp Val Asn Val
 385 390 395 400
 Glu Ile Val Phe Leu His Asn Ile Ser Pro Asn Leu Glu Leu Glu Ala
 405 410 415
 Leu Phe Lys Arg His Phe Thr Gln Val Glu Phe Tyr Gln Gly Ser Val
 420 425 430
 Leu Asn Pro His Asp Leu Ala Arg Val Lys Ile Glu Ser Ala Asp Ala
 435 440 445
 Cys Leu Ile Leu Ala Asn Lys Tyr Cys Ala Asp Pro Asp Ala Glu Asp
 450 455 460
 Ala Ser Asn Ile Met Arg Val Ile Ser Ile Lys Asn Tyr His Pro Lys
 465 470 475 480
 Ile Arg Ile Ile Thr Gln Met Leu Gln Tyr His Asn Lys Ala His Leu
 485 490 495
 Leu Asn Ile Pro Ser Trp Asn Trp Lys Glu Gly Asp Asp Ala Ile Cys
 500 505 510
 Leu Ala Glu Leu Lys Leu Gly Phe Ile Ala Gln Ser Cys Leu Ala Gln
 515 520 525
 Gly Leu Ser Thr Met Leu Ala Asn Leu Phe Ser Met Arg Ser Phe Ile
 530 535 540

Lys Ile Glu Glu Asp Thr Trp Gln Lys Tyr Tyr Leu Glu Gly Val Ser
 545 550 555 560
 Asn Glu Met Tyr Thr Glu Tyr Leu Ser Ser Ala Phe Val Gly Leu Ser
 565 570 575
 Phe Pro Thr Val Cys Glu Leu Cys Phe Val Lys Leu Lys Leu Leu Met
 580 585 590
 Ile Ala Ile Glu Tyr Lys Ser Ala Asn Arg Glu Ser Arg Ile Leu Ile
 595 600 605
 Asn Pro Gly Asn His Leu Lys Ile Gln Glu Gly Thr Leu Gly Phe Phe
 610 615 620
 Ile Ala Ser Asp Ala Lys Glu Val Lys Arg Ala Phe Phe Tyr Cys Lys
 625 630 635 640
 Ala Cys His Asp Asp Val Thr Asp Pro Lys Arg Ile Lys Lys Cys Gly
 645 650 655
 Cys Arg Arg Leu Ile Tyr Phe Glu Asp Glu Gln Pro Pro Thr Leu Ser
 660 665 670
 Pro Lys Lys Lys Gln Arg Asn Gly Gly Met Arg Asn Ser Pro Asn Thr
 675 680 685
 Ser Pro Lys Leu Met Arg His Asp Pro Leu Leu Ile Pro Gly Asn Asp
 690 695 700
 Gln Ile Asp Asn Met Asp Ser Asn Val Lys Lys Tyr Asp Ser Thr Gly
 705 710 715 720
 Met Phe His Trp Cys Ala Pro Lys Glu Ile Glu Lys Val Ile Leu Thr
 725 730 735
 Arg Ser Glu Ala Ala Met Thr Val Leu Ser Gly His Val Val Val Cys
 740 745 750
 Ile Phe Gly Asp Val Ser Ser Ala Leu Ile Gly Leu Arg Asn Leu Val
 755 760 765
 Met Pro Leu Arg Ala Ser Asn Phe His Tyr His Glu Leu Lys His Ile
 770 775 780
 Val Phe Val Gly Ser Ile Glu Tyr Leu Lys Arg Glu Trp Glu Thr Leu
 785 790 795 800
 His Asn Phe Pro Lys Val Ser Ile Leu Pro Gly Thr Pro Leu Ser Arg
 805 810 815
 Ala Asp Leu Arg Ala Val Asn Ile Asn Leu Cys Asp Met Cys Val Ile
 820 825 830
 Leu Ser Ala Asn Gln Asn Asn Ile Asp Asp Thr Ser Leu Gln Asp Lys
 835 840 845
 Glu Cys Ile Leu Ala Ser Leu Asn Ile Lys Ser Met Gln Phe Asp Asp
 850 855 860
 Ser Ile Gly Val Leu Gln Ala Asn Ser Gln Gly Phe Thr Pro Pro Gly
 865 870 875 880

Met Asp Arg Ser Ser Pro Asp Asn Ser Pro Val His Gly Met Leu Arg
 885 890 895
 Gln Pro Ser Ile Thr Thr Gly Val Asn Ile Pro Ile Ile Thr Glu Leu
 900 905 910
 Val Asn Asp Thr Asn Val Gln Phe Leu Asp Gln Asp Asp Asp Asp Asp
 915 920 925
 Pro Asp Thr Glu Leu Tyr Leu Thr Gln Pro Phe Ala Cys Gly Thr Ala
 930 935 940
 Phe Ala Val Ser Val Leu Asp Ser Leu Met Ser Ala Thr Tyr Phe Asn
 945 950 955 960
 Asp Asn Ile Leu Thr Leu Ile Arg Thr Leu Val Thr Gly Gly Ala Thr
 965 970 975
 Pro Glu Leu Glu Ala Leu Ile Ala Glu Glu Asn Ala Leu Arg Gly Gly
 980 985 990
 Tyr Ser Thr Pro Gln Thr Leu Ala Asn Arg Asp Arg Cys Arg Val Ala
 995 1000 1005
 Gln Leu Ala Leu Leu Asp Gly Pro Phe Ala Asp Leu Gly Asp Gly Gly
 1010 1015 1020
 Cys Tyr Gly Asp Leu Phe Cys Lys Ala Leu Lys Thr Tyr Asn Met Leu
 1025 1030 1035 1040
 Cys Phe Gly Ile Tyr Arg Leu Arg Asp Ala His Leu Ser Thr Pro Ser
 1045 1050 1055
 Gln Cys Thr Lys Arg Tyr Val Ile Thr Asn Pro Pro Tyr Glu Phe Glu
 1060 1065 1070
 Leu Val Pro Thr Asp Leu Ile Phe Cys Leu Met Gln Phe Asp His Asn
 1075 1080 1085
 Ala Gly Gln Ser Arg Ala Ser Leu Ser His Ser Ser His Ser Ser Gln
 1090 1095 1100
 Ser Ser Ser Lys Lys Ser Ser Ser Val His Ser Ile Pro Ser Thr Ala
 1105 1110 1115 1120
 Asn Arg Pro Asn Arg Pro Lys Ser Arg Glu Ser Arg Asp Lys Gln Asn
 1125 1130 1135
 Ala Thr Arg Met Thr Arg Met Gly Gln Ala Glu Lys Lys Trp Phe Thr
 1140 1145 1150
 Asp Glu Pro Asp Asn Ala Tyr Pro Arg Asn Ile Gln Ile Lys Pro Met
 1155 1160 1165
 Ser Thr His Met Ala Asn Gln Ile Asn Gln Tyr Lys Ser Thr Ser Ser
 1170 1175 1180
 Leu Ile Pro Pro Ile Arg Glu Val Glu Asp Glu Cys
 1185 1190 1195

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 731 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met	Asp	Thr	Ser	Gly	His	Phe	His	Asp	Ser	Gly	Val	Gly	Asp	Leu	Asp
1															15
Glu	Asp	Pro	Lys	Cys	Pro	Cys	Pro	Ser	Ser	Gly	Asp	Glu	Gln	Gln	Gln
															30
Gln	Pro	Pro	Pro	Pro	Ala	Ser	Pro								
															45
Ala	Ala	Pro	Gln	Gln	Pro	Leu	Gly	Pro	Ser	Leu	Gln	Pro	Gln	Pro	Pro
															60
Gln	Leu	Gln													
65															80
Ser	Pro	His	Pro	Leu	Ser	Gln	Leu	Ala	Gln	Leu	Gln	Ser	Gln	Pro	Val
															95
His	Pro	Gly	Leu	Leu	His	Ser	Ser	Pro	Thr	Ala	Phe	Arg	Ala	Pro	Pro
															110
Ser	Ser	Asn	Ser	Thr	Ala	Ile	Leu	His	Pro	Ser	Ser	Arg	Gln	Gly	Ser
															125
Gln	Leu	Asn	Leu	Asn	Asp	His	Leu	Leu	Gly	His	Ser	Pro	Ser	Ser	Thr
															140
Ala	Thr	Ser	Gly	Pro	Gly	Gly	Ser	Arg	His	Arg	Gln	Ala	Ser	Pro	
															160
Leu	Val	His	Arg	Arg	Asp	Ser	Asn	Pro	Phe	Thr	Glu	Ile	Ala	Met	Ser
															175
Ser	Cys	Lys	Tyr	Ser	Gly	Gly	Val	Met	Lys	Pro	Leu	Ser	Arg	Phe	Ser
															190
Ala	Ser	Arg	Arg	Asn	Leu	Ile	Glu	Ala	Glu	Thr	Glu	Gly	Gln	Pro	Leu
															205
Gln	Leu	Phe	Ser	Pro	Ser	Asn	Pro	Pro	Glu	Ile	Val	Ile	Ser	Ser	Arg
															220
Glu	Asp	Asn	His	Ala	His	Gln	Thr	Leu	Leu	His	His	Pro	Asn	Ala	Thr
															240
His	Asn	His	Gln	His	Ala	Gly	Thr	Thr	Ala	Ser	Ser	Thr	Thr	Phe	Pro
															255

Lys Ala Asn Lys Arg Lys Asn Gln Asn Ile Gly Tyr Lys Leu Gly His
 260 265 270
 Arg Arg Ala Leu Phe Glu Lys Arg Lys Arg Leu Ser Asp Tyr Ala Leu
 275 280 285
 Ile Phe Gly Met Phe Gly Ile Val Val Met Val Ile Glu Thr Glu Leu
 290 295 300
 Ser Trp Gly Leu Tyr Ser Lys Asp Ser Met Phe Ser Leu Ala Leu Lys
 305 310 315 320
 Cys Arg Ile Ser Leu Ser Thr Ile Ile Leu Leu Gly Leu Ile Ile Ala
 325 330 335
 Tyr His Thr Arg Gly Val Gln Leu Phe Val Ile Asp Asn Asp Ala Asp
 340 345 350
 Asp Trp Arg Ile Ala Met Thr Tyr Glu Arg Ile Leu Tyr Ile Ser Leu
 355 360 365
 Glu Met Leu Val Tyr Thr Asn His Thr Ile Pro Gly Glu Tyr Lys Phe
 370 375 380
 Phe Trp Ala Ala Arg Leu Ala Phe Ser Tyr Thr Pro Ser Arg Ala Glu
 385 390 395 400
 Ala Asp Val Asp Ile Ile Leu Ser Ile Pro Met Phe Leu Arg Leu Tyr
 405 410 415
 Leu Ile Ala Arg Val Met Leu Leu His Ser Lys Leu Phe Thr Asp Ala
 420 425 430
 Ser Ser Arg Ser Ile Gly Ala Leu Asn Lys Ile Asn Phe Asn Thr Arg
 435 440 445
 Phe Val Met Lys Thr Leu Met Thr Ile Cys Pro Gly Thr Val Leu Leu
 450 455 460
 Val Phe Ser Ile Ser Leu Trp Ile Ile Ala Ala Trp Thr Val Arg Val
 465 470 475 480
 Cys Glu Arg Tyr His Asp Gln Gln Asp Val Thr Ser Asn Phe Leu Gly
 485 490 495
 Ala Met Trp Leu Ile Ser Ile Thr Phe Leu Ser Ile Gly Tyr Gly Asp
 500 505 510
 Met Val Pro His Thr Tyr Cys Gly Lys Gly Val Cys Leu Leu Thr Gly
 515 520 525
 Ile Met Gly Ala Gly Cys Thr Ala Leu Val Val Ala Val Val Ala Arg
 530 535 540
 Lys Leu Glu Leu Thr Lys Ala Glu Lys His Val Asp Asn Phe Met Met
 545 550 555 560
 Asp Thr Gln Leu Thr Lys Arg Ile Lys Asn Ala Ala Ala Asn Val Leu
 565 570 575
 Arg Glu Thr Trp Leu Ile Tyr Lys His Thr Lys Leu Leu Lys Lys Ile
 580 585 590
 Asp His Ala Lys Val Arg Lys His Gln Arg Lys Phe Leu Gln Ala Ile

595	600	605
His Gln Leu Arg Ser Val Lys Met Glu Gln Arg Lys Leu Ser Asp Gln		
610	615	620
Ala Asn Thr Leu Val Asp Leu Ser Lys Met Gln Asn Val Met Tyr Asp		
625	630	635
Leu Ile Thr Glu Leu Asn Asp Arg Ser Glu Asp Leu Glu Lys Gln Ile		
645	650	655
Gly Ser Leu Glu Ser Lys Leu Glu His Leu Thr Ala Ser Phe Asn Ser		
660	665	670
Leu Pro Leu Leu Ile Ala Asp Thr Leu Arg Gln Gln Gln Gln Leu		
675	680	685
Leu Ser Ala Ile Ile Glu Ala Arg Gly Val Ser Val Ala Val Gly Thr		
690	695	700
Thr His Thr Pro Ile Ser Asp Thr Pro Ile Gly Val Ser Ser Thr Ser		
705	710	715
Phe Pro Thr Pro Tyr Thr Ser Ser Ser Cys		
725	730	730

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 857 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Arg Gly Gly Ala Leu Leu Cys Gly Gln Val Gln Asp Glu Ile Glu			
1	5	10	15
Gln Leu Ser Arg Glu Ser Ser His Phe Ser Leu Ser Thr Gly Ile Leu			
20	25	30	
Pro Ser Leu Gly Ala Arg Ser Asn Arg Arg Val Lys Leu Arg Arg Phe			
35	40	45	
Val Val Ser Pro Tyr Asp His Lys Tyr Arg Ile Trp Glu Ala Phe Leu			
50	55	60	
Val Val Leu Val Val Tyr Thr Ala Trp Val Ser Pro Phe Glu Phe Gly			
65	70	75	80
Phe Leu Arg Lys Pro Arg Pro Pro Leu Ser Ile Thr Asp Asn Ile Val			
85	90	95	
Asn Ala Phe Phe Ala Ile Asp Ile Ile Met Thr Phe Phe Val Gly Tyr			
100	105	110	

Leu Asp Lys Ser Thr Tyr Leu Ile Val Asp Asp Arg Lys Gln Ile Ala
 115 120 125
 Phe Lys Tyr Leu Arg Ser Trp Phe Leu Leu Asp Leu Val Ser Thr Ile
 130 135 140
 Pro Ser Glu Ala Ala Met Arg Ile Ser Ser Gln Ser Tyr Gly Leu Phe
 145 150 155 160
 Asn Met Leu Arg Leu Trp Arg Leu Arg Arg Val Gly Ala Leu Phe Ala
 165 170 175
 Arg Leu Glu Lys Asp Arg Asn Phe Asn Tyr Phe Trp Val Arg Cys Ala
 180 185 190
 Lys Leu Val Cys Val Thr Leu Phe Ala Val His Cys Ala Ala Cys Phe
 195 200 205
 Tyr Tyr Leu Ile Ala Ala Arg Asn Ser Asn Pro Ala Lys Thr Trp Ile
 210 215 220
 Gly Ala Asn Val Ala Asn Phe Leu Glu Glu Ser Leu Trp Met Arg Tyr
 225 230 235 240
 Val Thr Ser Met Tyr Trp Ser Ile Thr Thr Leu Thr Thr Val Gly Tyr
 245 250 255
 Gly Asp Leu His Pro Val Asn Thr Lys Glu Met Ile Phe Asp Ile Phe
 260 265 270
 Tyr Met Leu Phe Asn Leu Gly Leu Thr Ala Tyr Leu Ile Gly Asn Met
 275 280 285
 Thr Asn Leu Val Val His Gly Thr Ser Arg Thr Arg Asn Phe Arg Asp
 290 295 300
 Thr Ile Gln Ala Ala Ser Asn Phe Ala His Arg Asn His Leu Pro Pro
 305 310 315 320
 Arg Leu Gln Asp Gln Met Leu Ala His Leu Cys Leu Lys Tyr Arg Thr
 325 330 335
 Asp Ser Glu Gly Leu Gln Gln Glu Thr Leu Asp Ala Leu Pro Lys
 340 345 350
 Ala Ile Arg Ser Ser Ile Ser His Phe Leu Phe Tyr Ser Leu Met Asp
 355 360 365
 Lys Val Tyr Leu Phe Arg Gly Val Ser Asn Asp Leu Leu Phe Gln Leu
 370 375 380
 Val Ser Glu Met Lys Ala Glu Tyr Phe Pro Pro Lys Glu Asp Val Ile
 385 390 395 400
 Leu Gln Asn Glu Ala Pro Thr Asp Phe Tyr Ile Leu Val Asn Gly Thr
 405 410 415
 Ala Asp Leu Val Asp Val Asp Thr Gly Thr Glu Ser Ile Val Arg Glu
 420 425 430
 Val Lys Ala Gly Asp Ile Ile Gly Glu Ile Gly Val Leu Cys Tyr Arg
 435 440 445

Pro Gln Leu Phe Thr Val Arg Thr Lys Arg Leu Cys Gln Leu Leu Arg
 450 455 460
 Met Asn Arg Thr Thr Phe Leu Asn Ile Ile Gln Ala Asn Val Gly Asp
 465 470 475 480
 Gly Thr Ile Ile Met Asn Asn Leu Leu Gln His Leu Lys Glu Met Asn
 485 490 495
 Asp Pro Val Met Thr Asn Val Leu Leu Glu Ile Glu Asn Met Leu Ala
 500 505 510
 Arg Gly Lys Met Asp Leu Pro Leu Asn Leu Cys Phe Ala Ala Ile Arg
 515 520 525
 Glu Asp Asp Leu Leu Leu His Gln Leu Leu Lys Arg Gly Leu Asp Pro
 530 535 540
 Asn Glu Ser Asp Asn Asn Gly Arg Thr Pro Leu His Ile Ala Ala Ser
 545 550 555 560
 Lys Gly Thr Leu Asn Cys Val Leu Leu Leu Glu Tyr His Ala Asp
 565 570 575
 Pro Asn Cys Arg Asp Ala Glu Gly Ser Val Pro Leu Trp Glu Ala Met
 580 585 590
 Val Glu Gly His Glu Lys Val Val Lys Val Leu Leu Glu His Gly Ser
 595 600 605
 Thr Ile Asp Ala Gly Asp Val Gly His Phe Ala Cys Thr Ala Ala Glu
 610 615 620
 Gln Gly Asn Leu Lys Leu Leu Lys Glu Ile Val Leu His Gly Gly Asp
 625 630 635 640
 Val Thr Arg Pro Arg Ala Thr Gly Thr Ser Ala Leu His Thr Ala Val
 645 650 655
 Cys Glu Glu Asn Ile Glu Met Val Lys Tyr Leu Leu Glu Gln Gly Ala
 660 665 670
 Asp Val Asn Lys Gln Asp Met His Gly Trp Thr Pro Arg Asp Leu Ala
 675 680 685
 Glu Gln Gln Gly His Glu Asp Ile Lys Ala Leu Phe Arg Glu Lys Leu
 690 695 700
 His Glu Arg Arg Val His Ile Glu Thr Ser Ser Ser Val Pro Ile Leu
 705 710 715 720
 Lys Thr Gly Ile Arg Phe Leu Gly Arg Phe Thr Ser Glu Pro Asn Ile
 725 730 735
 Arg Pro Ala Ser Arg Glu Val Ser Phe Arg Ile Arg Glu Thr Arg Ala
 740 745 750
 Arg Arg Lys Thr Asn Asn Phe Asp Asn Ser Leu Phe Gly Ile Leu Ala
 755 760 765
 Asn Gln Ser Val Pro Lys Asn Gly Leu Ala Thr Val Asp Glu Gly Arg
 770 775 780
 Thr Gly Asn Pro Val Arg Val Thr Ile Ser Cys Ala Glu Lys Asp Asp

785	790	795	800
Ile Ala Gly Lys Leu Val Leu Leu Pro Gly Ser Phe Lys Glu Leu Leu			
805		810	815
Glu Leu Gly Ser Asn Lys Phe Gly Ile Val Ala Thr Lys Val Met Asn			
820		825	830
Lys Asp Asn Asn Ala Glu Ile Asp Asp Val Asp Val Ile Arg Asp Gly			
835		840	845
Asp His Leu Ile Phe Ala Thr Asp Ser			
850		855	

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1159 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Pro Val Arg Arg Gly His Val Ala Pro Gln Asn Thr Phe Leu Asp				
1	5	10	15	
Thr Ile Ile Arg Lys Phe Glu Gly Gln Ser Arg Lys Phe Ile Ile Ala				
20		25	30	
Asn Ala Arg Val Glu Asn Cys Ala Val Ile Tyr Cys Asn Asp Gly Phe				
35		40	45	
Cys Glu Leu Cys Gly Tyr Ser Arg Ala Glu Val Met Gln Arg Pro Cys				
50		55	60	
Thr Cys Asp Phe Leu His Gly Pro Arg Thr Gln Arg Arg Ala Ala Ala				
65		70	75	80
Gln Ile Ala Gln Ala Leu Leu Gly Ala Glu Glu Arg Lys Val Glu Ile				
85		90	95	
Ala Phe Tyr Arg Lys Asp Gly Ser Cys Phe Leu Cys Leu Val Asp Val				
100		105	110	
Val Pro Val Lys Asn Glu Asp Gly Ala Val Ile Met Phe Ile Leu Asn				
115		120	125	
Phe Glu Val Val Met Glu Lys Asp Met Val Gly Ser Pro Ala His Asp				
130		135	140	
Thr Asn His Arg Gly Pro Pro Thr Ser Trp Leu Ala Pro Gly Arg Ala				
145		150	155	160
Lys Thr Phe Arg Leu Lys Leu Pro Ala Leu Leu Ala Leu Thr Ala Arg				
165		170	175	

Glu Ser Ser Val Arg Ser Gly Gly Ala Gly Gly Ala Pro Gly
 180 185 190
 Ala Val Val Val Asp Val Asp Leu Thr Pro Ala Ala Pro Ser Ser Glu
 195 200 205
 Ser Leu Ala Leu Asp Glu Val Thr Ala Met Asp Asn His Val Ala Gly
 210 215 220
 Leu Gly Pro Ala Glu Glu Arg Arg Ala Leu Val Gly Pro Gly Ser Pro
 225 230 235 240
 Pro Arg Ser Ala Pro Gly Gln Leu Pro Ser Pro Arg Ala His Ser Leu
 245 250 255
 Asn Pro Asp Ala Ser Gly Ser Ser Cys Ser Leu Ala Arg Thr Arg Ser
 260 265 270
 Arg Glu Ser Cys Ala Ser Val Arg Arg Ala Ser Ser Ala Asp Asp Ile
 275 280 285
 Glu Ala Met Arg Ala Gly Val Leu Pro Pro Pro Arg His Ala Ser
 290 295 300
 Thr Gly Ala Met His Pro Leu Arg Ser Gly Leu Leu Asn Ser Thr Ser
 305 310 315 320
 Asp Ser Asp Leu Val Arg Tyr Arg Thr Ile Ser Lys Ile Pro Gln Ile
 325 330 335
 Thr Leu Asn Phe Val Asp Leu Lys Gly Asp Pro Phe Leu Ala Ser Pro
 340 345 350
 Thr Ser Asp Arg Glu Ile Ile Ala Pro Lys Ile Lys Glu Arg Thr His
 355 360 365
 Asn Val Thr Glu Lys Val Thr Gln Val Leu Ser Leu Gly Ala Asp Val
 370 375 380
 Leu Pro Glu Tyr Lys Leu Gln Ala Pro Arg Ile His Arg Trp Thr Ile
 385 390 395 400
 Leu His Tyr Ser Pro Phe Lys Ala Val Trp Asp Trp Leu Ile Leu Leu
 405 410 415
 Leu Val Ile Tyr Thr Ala Val Phe Thr Pro Tyr Ser Ala Ala Phe Leu
 420 425 430
 Leu Lys Glu Thr Glu Glu Gly Pro Pro Ala Thr Glu Cys Gly Tyr Ala
 435 440 445
 Cys Gln Pro Leu Ala Val Val Asp Leu Ile Val Asp Ile Met Phe Ile
 450 455 460
 Val Asp Ile Leu Ile Asn Phe Arg Thr Thr Tyr Val Asn Ala Asn Glu
 465 470 475 480
 Glu Val Val Ser His Pro Gly Arg Ile Ala Val His Tyr Phe Lys Gly
 485 490 495
 Trp Phe Leu Ile Asp Met Val Ala Ala Ile Pro Phe Asp Leu Leu Ile
 500 505 510

Phe Gly Ser Gly Ser Glu Glu Leu Ile Gly Leu Leu Lys Thr Ala Arg
 515 520 525
 Leu Leu Arg Leu Val Arg Val Ala Arg Lys Leu Asp Arg Tyr Ser Glu
 530 535 540
 Tyr Gly Ala Ala Val Leu Phe Leu Leu Met Cys Thr Phe Ala Leu Ile
 545 550 555 560
 Ala His Trp Leu Ala Cys Ile Trp Tyr Ala Ile Gly Asn Met Glu Gln
 565 570 575
 Pro His Met Asp Ser Arg Ile Gly Trp Leu His Asn Leu Gly Asp Gln
 580 585 590
 Ile Gly Lys Pro Tyr Asn Ser Ser Gly Leu Gly Gly Pro Ser Ile Lys
 595 600 605
 Asp Lys Tyr Val Thr Ala Leu Tyr Phe Thr Phe Ser Ser Leu Thr Ser
 610 615 620
 Val Gly Phe Gly Asn Val Ser Pro Asn Thr Asn Ser Glu Lys Ile Phe
 625 630 635 640
 Ser Ile Cys Val Met Leu Ile Gly Ser Leu Met Tyr Ala Ser Ile Phe
 645 650 655
 Gly Asn Val Ser Ala Ile Ile Gln Arg Leu Tyr Ser Gly Thr Ala Arg
 660 665 670
 Tyr His Thr Gln Met Leu Arg Val Arg Glu Phe Ile Arg Phe His Gln
 675 680 685
 Ile Pro Asn Pro Leu Arg Gln Arg Leu Glu Glu Tyr Phe Gln His Ala
 690 695 700
 Trp Ser Tyr Thr Asn Gly Ile Asp Met Asn Ala Val Leu Lys Gly Phe
 705 710 715 720
 Pro Glu Cys Leu Gln Ala Asp Ile Cys Leu His Leu Asn Arg Ser Leu
 725 730 735
 Leu Gln His Cys Lys Pro Phe Arg Gly Ala Thr Lys Gly Cys Leu Arg
 740 745 750
 Ala Leu Ala Met Lys Phe Lys Thr Thr His Ala Pro Pro Gly Asp Thr
 755 760 765
 Leu Val His Ala Gly Asp Leu Leu Thr Ala Leu Tyr Phe Ile Ser Arg
 770 775 780
 Gly Ser Ile Glu Ile Leu Arg Gly Asp Val Val Val Ala Ile Leu Gly
 785 790 795 800
 Lys Asn Asp Ile Phe Gly Glu Pro Leu Asn Leu Tyr Ala Arg Pro Gly
 805 810 815
 Lys Ser Asn Gly Asp Val Arg Ala Leu Thr Tyr Cys Asp Leu His Lys
 820 825 830
 Ile His Arg Asp Asp Leu Leu Glu Val Leu Asp Met Tyr Pro Glu Phe
 835 840 845
 Ser Asp His Phe Trp Ser Ser Leu Glu Ile Thr Phe Asn Leu Arg Asp

850	855	860
Thr Asn Met Ile Pro Gly Ser Pro Gly Ser Thr Glu Leu Glu Gly Gly		
865	870	875
Phe Ser Arg Gln Arg Lys Arg Lys Leu Ser Phe Arg Arg Arg Thr Asp		
885	890	895
Lys Asp Thr Glu Gln Pro Gly Glu Val Ser Ala Leu Gly Pro Gly Arg		
900	905	910
Ala Gly Ala Gly Pro Ser Ser Arg Gly Arg Pro Gly Gly Pro Trp Gly		
915	920	925
Glu Ser Pro Ser Ser Gly Pro Ser Ser Pro Glu Ser Ser Glu Asp Glu		
930	935	940
Gly Pro Gly Arg Ser Ser Ser Pro Leu Arg Leu Val Pro Phe Ser Ser		
945	950	955
Pro Arg Pro Pro Gly Glu Pro Pro Gly Gly Glu Pro Leu Met Glu Asp		
965	970	975
Cys Glu Lys Ser Ser Asp Thr Cys Asn Pro Leu Ser Gly Ala Phe Ser		
980	985	990
Gly Val Ser Asn Ile Phe Ser Phe Trp Gly Asp Ser Arg Gly Arg Gln		
995	1000	1005
Tyr Gln Glu Leu Pro Arg Cys Pro Ala Pro Thr Pro Ser Leu Leu Asn		
1010	1015	1020
Ile Pro Leu Ser Ser Pro Gly Arg Arg Pro Arg Gly Asp Val Glu Ser		
1025	1030	1035
Arg Leu Asp Ala Leu Gln Arg Gln Leu Asn Arg Leu Glu Thr Arg Leu		
1045	1050	1055
Ser Ala Asp Met Ala Thr Val Leu Gln Leu Leu Gln Arg Gln Met Thr		
1060	1065	1070
Leu Val Pro Pro Ala Tyr Ser Ala Val Thr Thr Pro Gly Pro Gly Pro		
1075	1080	1085
Thr Ser Thr Ser Pro Leu Leu Pro Val Ser Pro Leu Pro Thr Leu Thr		
1090	1095	1100
Leu Asp Ser Leu Ser Gln Val Ser Gln Phe Met Ala Cys Glu Glu Leu		
1105	1110	1115
Pro Pro Gly Ala Pro Glu Leu Pro Gln Glu Gly Pro Thr Arg Arg Leu		
1125	1130	1135
Ser Leu Pro Gly Gln Leu Gly Ala Leu Thr Ser Gln Pro Leu His Arg		
1140	1145	1150
His Gly Ser Asp Pro Gly Ser		
1155		

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 391 amino acids

(B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Met	Gly	Ala	Ser	Glu	Arg	Ser	Val	Phe	Arg	Val	Leu	Ile	Arg	Ala	Leu
1				5						10			15		
Thr	Glu	Arg	Met	Phe	Lys	His	Leu	Arg	Arg	Trp	Phe	Ile	Thr	His	Ile
	20					25						30			
Phe	Gly	Arg	Ser	Arg	Gln	Arg	Ala	Arg	Leu	Val	Ser	Lys	Glu	Gly	Arg
	35				40							45			
Cys	Asn	Ile	Glu	Phe	Gly	Asn	Val	Asp	Ala	Gln	Ser	Arg	Phe	Ile	Phe
	50				55				60						
Phe	Val	Asp	Ile	Trp	Thr	Thr	Val	Leu	Asp	Leu	Lys	Trp	Arg	Tyr	Lys
	65				70			75			80				
Met	Thr	Val	Phe	Ile	Thr	Ala	Phe	Leu	Gly	Ser	Trp	Phe	Leu	Phe	Gly
		85				90				95					
Leu	Leu	Trp	Tyr	Val	Val	Ala	Tyr	Val	His	Lys	Asp	Leu	Pro	Glu	Phe
		100			105				110						
Tyr	Pro	Pro	Asp	Asn	Arg	Thr	Pro	Cys	Val	Glu	Asn	Ile	Asn	Gly	Met
	115				120					125					
Thr	Ser	Ala	Phe	Leu	Phe	Ser	Leu	Glu	Thr	Gln	Val	Thr	Ile	Gly	Tyr
	130				135				140						
Gly	Phe	Arg	Phe	Val	Thr	Glu	Gln	Cys	Ala	Thr	Ala	Ile	Phe	Leu	Leu
	145				150			155			160				
Ile	Phe	Gln	Ser	Ile	Leu	Gly	Val	Ile	Ile	Asn	Ser	Phe	Met	Cys	Gly
	165				170			175							
Ala	Ile	Leu	Ala	Lys	Ile	Ser	Arg	Pro	Lys	Lys	Arg	Ala	Lys	Thr	Ile
	180				185			190							
Thr	Phe	Ser	Lys	Asn	Ala	Val	Ile	Ser	Lys	Arg	Gly	Gly	Lys	Leu	Cys
	195				200			205							
Leu	Leu	Ile	Arg	Val	Ala	Asn	Leu	Arg	Lys	Ser	Leu	Leu	Ile	Gly	Ser
	210				215			220							
His	Ile	Tyr	Gly	Lys	Leu	Leu	Lys	Thr	Thr	Ile	Thr	Pro	Glu	Gly	Glu
	225				230			235		240					
Thr	Ile	Ile	Leu	Asp	Gln	Thr	Asn	Ile	Asn	Phe	Val	Val	Asp	Ala	Gly
	245				250			250		255					
Asn	Glu	Asn	Leu	Phe	Phe	Ile	Ser	Pro	Leu	Thr	Ile	Tyr	His	Ile	Ile
	260				265			265		270					

Asp His Asn Ser Pro Phe Phe His Met Ala Ala Glu Thr Leu Ser Gln			
275	280	285	
Gln Asp Phe Glu Leu Val Val Phe Leu Asp Gly Thr Val Glu Ser Thr			
290	295	300	
Ser Ala Thr Cys Gln Val Arg Thr Ser Tyr Val Pro Glu Glu Val Leu			
305	310	315	320
Trp Gly Tyr Arg Phe Val Pro Ile Val Ser Lys Thr Lys Glu Gly Lys			
325	330	335	
Tyr Arg Val Asp Phe His Asn Phe Gly Lys Thr Val Glu Val Glu Thr			
340	345	350	
Pro His Cys Ala Met Cys Leu Tyr Asn Glu Lys Asp Ala Arg Ala Arg			
355	360	365	
Met Lys Arg Gly Tyr Asp Asn Pro Asn Phe Val Leu Ser Glu Val Asp			
370	375	380	
Glu Thr Asp Asp Thr Gln Met			
385	390		

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 407 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Asp Gln Asp Val Glu Ser Pro Val Ala Ile His Gln Pro Lys Leu			
1	5	10	15
Pro Lys Gln Ala Arg Asp Asp Leu Pro Arg His Ile Ser Arg Asp Arg			
20	25	30	
Thr Lys Arg Lys Ile Gln Arg Tyr Val Arg Lys Asp Gly Lys Cys Asn			
35	40	45	
Val His His Gly Asn Val Arg Glu Thr Tyr Arg Tyr Leu Thr Asp Ile			
50	55	60	
Phe Thr Thr Leu Val Asp Leu Lys Trp Arg Phe Asn Leu Leu Ile Phe			
65	70	75	80
Val Met Val Tyr Thr Val Thr Trp Leu Phe Phe Gly Met Ile Trp Trp			
85	90	95	
Leu Ile Ala Tyr Ile Arg Gly Asp Met Asp His Ile Glu Asp Ser Pro			
100	105	110	

Trp Thr Pro Cys Val Thr Asn Leu Asn Gly Phe Val Ser Ala Phe Leu
 115 120 125
 Phe Ser Ile Glu Thr Glu Thr Thr Ile Gly Tyr Gly Tyr Arg Val Ile
 130 135 140
 Thr Asp Lys Cys Pro Glu Gly Ile Ile Leu Leu Ile Gln Ser Val
 145 150 155 160
 Leu Gly Ser Ile Val Asn Ala Phe Met Val Gly Cys Met Phe Val Lys
 165 170 175
 Ile Ser Gln Pro Lys Lys Arg Ala Glu Thr Leu Val Phe Ser Thr His
 180 185 190
 Ala Val Ile Ser Met Arg Asp Gly Lys Leu Cys Leu Met Phe Arg Val
 195 200 205
 Gly Asp Leu Arg Asn Ser His Ile Val Glu Ala Ser Ile Arg Ala Lys
 210 215 220
 Leu Ile Lys Ser Lys Gln Thr Ser Glu Gly Glu Phe Ile Pro Leu Asn
 225 230 235 240
 Gln Thr Asp Ile Asn Val Gly Tyr Tyr Thr Gly Asp Asp Arg Leu Phe
 245 250 255
 Leu Val Ser Pro Leu Ile Ile Ser His Glu Ile Asn Gln Gln Ser Pro
 260 265 270
 Phe Trp Glu Ile Ser Lys Ala Gln Leu Pro Lys Glu Glu Leu Glu Ile
 275 280 285
 Val Val Ile Leu Glu Gly Met Val Glu Ala Thr Gly Met Thr Cys Gln
 290 295 300
 Ala Arg Ser Ser Tyr Ile Thr Ser Glu Ile Leu Trp Gly Tyr Arg Phe
 305 310 315 320
 Thr Pro Val Leu Thr Leu Glu Asp Gly Phe Tyr Glu Val Asp Tyr Asn
 325 330 335
 Ser Phe His Glu Thr Tyr Glu Thr Ser Thr Pro Ser Leu Ser Ala Lys
 340 345 350
 Glu Leu Ala Glu Leu Ala Ser Arg Ala Glu Leu Pro Leu Ser Trp Ser
 355 360 365
 Val Ser Ser Lys Leu Asn Gln His Ala Glu Leu Glu Thr Glu Glu Glu
 370 375 380
 Glu Lys Asn Leu Glu Glu Gln Thr Glu Arg Asn Gly Asp Val Ala Asn
 385 390 395 400
 Leu Glu Asn Glu Ser Lys Val
 405

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 5 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Thr Val Tyr Gly Asp
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 160 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met Pro Pro Met Leu Ser Gly Leu Leu Ala Arg Leu Val Lys Leu Leu
1 5 10 15

Leu Gly Arg His Gly Ser Ala Leu His Trp Arg Ala Ala Gly Ala Ala
20 25 30

Thr Val Leu Leu Val Ile Val Leu Leu Ala Gly Ser Tyr Leu Ala Val
35 40 45

Leu Ala Glu Arg Gly Ala Pro Gly Ala Ala Leu Ile Ser Tyr Pro Asp
50 55 60

Ala Leu Trp Trp Ser Val Glu Thr Ala Thr Thr Val Gly Tyr Gly Asp
65 70 75 80

Leu Tyr Pro Val Thr Leu Trp Gly Arg Leu Val Ala Val Val Val Met
85 90 95

Val Ala Gly Ile Thr Ser Phe Gly Leu Val Thr Ala Ala Leu Ala Thr
100 105 110

Trp Phe Val Gly Arg Glu Gln Glu Arg Arg Gly His Phe Val Arg His
115 120 125

Ser Glu Lys Ala Ala Glu Glu Ala Tyr Thr Arg Thr Thr Arg Ala Leu
130 135 140

His Glu Arg Phe Asp Arg Leu Glu Arg Met Leu Asp Asp Asn Arg Arg
145 150 155 160

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1161 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCATGCTGGC	TCCTTTGGGA	TCGATCCGTC	CGGTTCTTCT	CCGGCCGGCC	ACCTCTCGAA	60
GGTGACGCTG	TCGCCGACGA	GCCACCGACA	TCCGACCGAC	AGCCCCCGAC	AGCGCTCCTA	120
CGCGGTGCCG	ACATGACACC	GACACCGCAG	GTCGGACGAC	GGGGGCTCAG	GCGCGACGGG	180
CGCGGATCAC	GACGGCCGTA	CCGCGCGAC	GGCGAGCACC	GCCGCGCCGC	CGAGGAGTGG	240
CCGAAGGAGT	GAAGATCGGT	TACGGACCGT	AAAGGAGTAC	CTGGCGCACC	GGCGCGTTGT	300
CGCATCGTCG	TCCC GGCGGG	TGGCGGAGCA	TGCCACCCAT	GCTGTCCGGT	CTTCTGGCCA	360
GATTGGTCAA	ACTGCTGCTC	GGGCGCCACG	GCAGTGCCT	GCACTGGAGG	GCCGCGGGTG	420
CCGCGACGGT	CCTCCTGGTG	ATCGTCCCTCC	TCGCGGGCTC	GTACTTGGCC	GTCCTGGCTG	480
AGCGCGCGC	ACCGGGCGCG	GCGCTGATCT	CGTATCCGGA	CGCGCTGTGG	TGGTCCGTGG	540
AGACCGCGAC	GACCGTCGGC	TACGGCGACC	TGTACCCCGT	GACTCTGTGG	GGCCGGCTCG	600
TGGCCGTGGT	GGTGATGGTC	GCCGGGATCA	CCTCCTTCGG	TCTGGTGACC	GCCGCGCTGG	660
CCACCTGGTT	CGTCGGCCGG	GAACAAGAGC	GCCGGGGCCA	CTTCGTGCGC	CACTCCGAGA	720
AGGCCGCCGA	GGAGGGCGTAC	ACGCGGACGA	CCCAGGCGCT	GCACGAGCGT	TTCGACCGTT	780
TGGAGCGAAT	GCTCGACGAC	AACCGCCCGT	GACTCCGCCG	GTGACCGCCC	GAGCGAGGCC	840
GCACCGATGA	GTCTGCGGCG	GTTGTGCGGT	CTACCCGTG	ACGAAGGGAG	CGCACCATGC	900
GCAAGATCAT	CATTTGCACG	TTCCCTGACGC	TGGACGGCGT	CATGCAGGCG	CCGGCGGCC	960
CGGACGAGGA	CGCCGAGAGC	GGCTTCGAAC	ACGGCGGCTG	GCAGAAGCCG	GTGGACGACG	1020
ACGAGGTCGG	CACGGCCATC	GCCGGCTGGT	ACGAGGACTC	CGACGCCATG	CTCCTCGGCC	1080
GCAAGACCTA	CGACATCTTC	GCGTCGTACT	GGCCGACCGC	CGACCCCGAC	AACCCGTTCA	1140
CCCATCGGAT	GAACAGCATG	C				1161

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 261 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Val Tyr Pro Asn Ile Thr Asp Pro Glu Tyr Gly Tyr Leu Ala Arg Glu
20 25 30

Tyr Ile Tyr Cys Leu Tyr Trp Ser Thr Leu Thr Leu Thr Thr Ile Gly
 35 40 45

Glu Thr Pro Pro Pro Val Lys Asp Glu Glu Tyr Leu Phe Val Ile Phe
50 55 60

Asp Phe Leu Ile Gly Val Leu Ile Phe Ala Thr Ile Val Gly Asn Val
65 70 75 80

Gly Ser Met Ile Ser Asn Met Asn Ala Thr Arg Ala Glu Phe Gln Ala
85 90 95

Lys Ile Asp Ala Val Lys His Tyr Met Gln Phe Arg Lys Val Ser Lys
100 105 110

Gly Met Glu Ala Lys Val Ile Arg Trp Phe Asp Tyr Leu Trp Thr Asn
115 120 125

Lys Lys Thr Val Asp Glu Arg Glu Ile Leu Lys Asn Leu Pro Ala Lys
130 135 140

Leu Arg Ala Glu Ile Ala Ile Asn Val His Leu Ser Thr Leu Lys Lys
145 150 155 160

Val Arg Ile Phe His Asp Cys Glu Ala Gly Leu Leu Val Glu Leu Val
165 170 175

Leu Lys Leu Arg Pro Gln Val Phe Ser Pro Gly Asp Tyr Ile Cys Arg
180 185 190

Lys Gly Asp Ile Gly Lys Glu Met Tyr Ile Ile Lys Glu Gly Lys Leu
195 200 205

Ala Val Val Ala Asp Asp Gly Val Thr Gln Tyr Ala Leu Leu Ser Ala
210 215 220

Gly Ser Cys Phe Gly Glu Ile Ser Ile Leu Asn Ile Lys Gly Ser Lys
225 230 235 240

Met Gly Asn Arg Arg Thr Ala Asn Ile Arg Ser Leu Gly Tyr Ser Asp
245 250 255

Leu Phe Cys Leu Ser
260

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 690 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Met Lys Leu Ser Met Lys Asn Asn Ile Ile Asn Thr Gln Gln Ser Phe
1 5 10 15

Val Thr Met Pro Asn Val Ile Val Pro Asp Ile Glu Lys Glu Ile Arg
20 25 30

Arg Met Glu Asn Gly Ala Cys Ser Ser Phe Ser Glu Asp Asp Asp Ser
35 40 45

Ala Tyr Thr Ser Glu Glu Ser Glu Asn Glu Asn Pro His Ala Arg Gly
50 55 60

Ser Phe Ser Tyr Lys Ser Leu Arg Lys Gly Gly Pro Ser Gln Arg Glu
65 70 75 80

Gln Tyr Leu Pro Gly Ala Ile Ala Ile Phe Asn Val Asn Asn Ser Ser
85 90 95

Asn Lys Asp Gln Glu Pro Glu Glu Lys Lys Lys Lys Lys Glu Lys
100 105 110

Lys Ser Lys Ser Asp Asp Lys Asn Glu Asn Lys Asn Asp Pro Glu Lys
115 120 125

Lys Lys Lys Lys Asp Lys Glu Lys Lys Lys Glu Glu Lys Ser
130 135 140

Lys Asp Lys Lys Glu His His Lys Lys Glu Val Val Val Ile Asp Pro
145 150 155 160

Ser Gly Asn Thr Tyr Tyr Asn Trp Leu Phe Cys Ile Thr Leu Pro Val
165 170 175

Met Tyr Asn Trp Thr Met Val Ile Ala Arg Ala Cys Phe Asp Glu Leu
180 185 190

Gln Ser Asp Tyr Leu Glu Tyr Trp Leu Ile Leu Asp Tyr Val Ser Asp
195 200 205

Ile Val Tyr Leu Ile Asp M t Phe Val Arg Thr Arg Thr Gly Tyr Leu
210 215 220

Glu Gln Gly Leu Leu Val Lys Glu Glu Leu Lys Leu Ile Asn Lys Tyr
225 230 235 240

Lys Ser Asn Leu Gln Phe Lys Leu Asp Val Leu Ser Leu Ile Pro Thr
 245 250 255
 Asp Leu Leu Tyr Phe Lys Leu Gly Trp Asn Tyr Pro Glu Ile Arg Leu
 260 265 270
 Asn Arg Leu Leu Arg Phe Ser Arg Met Phe Glu Phe Phe Gln Arg Thr
 275 280 285
 Glu Thr Arg Thr Asn Tyr Pro Asn Ile Phe Arg Ile Ser Asn Leu Val
 290 295 300
 Met Tyr Ile Val Ile Ile His Trp Asn Ala Cys Val Phe Tyr Ser
 305 310 315 320
 Ile Ser Lys Ala Ile Gly Phe Gly Asn Asp Thr Trp Val Tyr Pro Asp
 325 330 335
 Ile Asn Asp Pro Glu Phe Gly Arg Leu Ala Arg Lys Tyr Val Tyr Ser
 340 345 350
 Leu Tyr Trp Ser Thr Leu Thr Leu Thr Ile Gly Glu Thr Pro Pro
 355 360 365
 Pro Val Arg Asp Ser Glu Tyr Val Phe Val Val Val Asp Phe Leu Ile
 370 375 380
 Gly Val Leu Ile Phe Ala Thr Ile Val Gly Asn Ile Gly Ser Met Ile
 385 390 395 400
 Ser Asn Met Asn Ala Ala Arg Ala Glu Phe Gln Ala Arg Ile Asp Ala
 405 410 415
 Ile Lys Gln Tyr Met His Phe Arg Asn Val Ser Lys Asp Met Glu Lys
 420 425 430
 Arg Val Ile Lys Trp Phe Asp Tyr Leu Trp Thr Asn Lys Lys Thr Val
 435 440 445
 Asp Glu Lys Glu Val Leu Lys Tyr Leu Pro Asp Lys Leu Arg Ala Glu
 450 455 460
 Ile Ala Ile Asn Val His Leu Asp Thr Leu Lys Lys Val Arg Ile Phe
 465 470 475 480
 Ala Asp Cys Glu Ala Gly Leu Leu Val Glu Leu Val Leu Lys Leu Gln
 485 490 495
 Pro Gln Val Tyr Ser Pro Gly Asp Tyr Ile Cys Lys Lys Gly Asp Ile
 500 505 510
 Gly Arg Glu Met Tyr Ile Ile Lys Glu Gly Lys Leu Ala Val Val Ala
 515 520 525
 Asp Asp Gly Val Thr Gln Phe Val Val Leu Ser Asp Gly Ser Thr Phe
 530 535 540
 Gly Glu Ile Ser Ile Leu Asn Ile Lys Gly Ser Lys Ala Gly Asn Arg
 545 550 555 560
 Arg Thr Ala Asn Ile Lys Ser Ile Gly Tyr Ser Asp Leu Phe Cys Leu
 565 570 575

Ser Lys Asp Asp Leu Met Glu Ala Leu Thr Glu Tyr Pro Asp Ala Lys
580 585 590

Thr Met Leu Glu Glu Lys Gly Lys Gln Ile Leu Met Lys Asp Gly Leu
595 600 605

Leu Asp Leu Asn Ile Ala Asn Ala Gly Ser Asp Pro Lys Asp Leu Glu
610 615 620

Glu Lys Val Thr Arg Met Glu Gly Ser Val Asp Leu Leu Gln Thr Arg
625 630 635 640

Phe Ala Arg Ile Leu Ala Glu Tyr Glu Ser Met Gln Gln Lys Leu Lys
645 650 655

Gln Arg Leu Thr Lys Val Glu Lys Phe Leu Lys Pro Leu Ile Asp Thr
660 665 670

Glu Phe Ser Ser Ile Glu Gly Pro Trp Ser Glu Ser Gly Pro Ile Asp
675 680 685

Ser Thr
690

Ser Lys Asp Asp Leu Met Glu Ala Leu Thr Glu Tyr Pro Asp Ala Lys
580 585 590

Thr Met Leu Glu Glu Lys Gly Lys Gln Ile Leu Met Lys Asp Gly Leu
595 600 605

Leu Asp Leu Asn Ile Ala Asn Ala Gly Ser Asp Pro Lys Asp Leu Glu
610 615 620

Glu Lys Val Thr Arg Met Glu Gly Ser Val Asp Leu Leu Gln Thr Arg
625 630 635 640

Phe Ala Arg Ile Leu Ala Glu Tyr Glu Ser Met Gln Gln Lys Leu Lys
645 650 655

Gln Arg Leu Thr Lys Val Glu Lys Phe Leu Lys Pro Leu Ile Asp Thr
660 665 670

Glu Phe Ser Ser Ile Glu Gly Pro Trp Ser Glu Ser Gly Pro Ile Asp
675 680 685

Ser Thr
690